THE VALUE OF HYDROGEN PEROXID IN THE MICROCHEMICAL DETERMINATION OF IRON.*

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PLATE LXII.

The use of hydrogen peroxid in the microchemical determination of certain forms of iron has proved of such great value to the author that he feels that the method deserves a wider application and should fill a definite need in our present technique.

Without considering in detail all the methods and their minor modifications which have been recommended for the microchemical determination of organic iron in tissues, it may be said that two general forms of technique are now in use.[1] One method is applicable to iron-containing substances, such as hemosiderin, which react directly when treated with ammonium sulphid or with potassium ferrocyanid, or ferricyanid, and hydrochloric acid. The other method is employed to convert the masked iron of such elements as cell nuclei into a form capable of manifesting a reaction with the above reagents. By the first method, all so-called albuminate iron will react, while by the second, the albuminate iron is usually removed from the section coincident with the liberation of the masked iron. By neither of these methods is a perfect composite picture obtainable. Although the ammonium sulphid glycerin method of Macallum[2] may possibly be employed to obtain an iron reaction from hemoglobin, certainly no method has been devised, that is at once reliable and easy of application, for the demonstration of iron in hemoglobin and its various modifications and such iron-containing derivatives as hemin and hematin.

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[1] For a detailed consideration of methods and literature, see Tracy, *Jour. Med. Research*, 1905–6, xiv, 1.

[2] *Proc. Roy. Soc. of London*, 1891, l, 277.

There is thus a large amount of tissue iron which by the present technique is with difficulty, or not at all, demonstrable, and there are possibly other forms than those that have been indicated. It was especially for the purpose of rendering demonstrable, in tissues, the iron of hemoglobin and its various modifications and iron-containing derivatives that the investigation of the action of oxidizing agents was undertaken.

It is a well known fact that when a pure solution of hemoglobin is treated with hydrogen peroxid, it is rapidly decolorized and decomposed with the production of a flocculent precipitate and the liberation of the iron in such a form that it can be detected by any of the usual tests for iron. It is equally well established that hematin and hemin are amenable to destructive oxidation by hydrogen peroxid, as well as by other methods of oxidation, with the liberation of the iron in a demonstrable form. On the other hand, nothing definite is reported of the changes produced in fixed red blood corpuscles by treatment with hydrogen peroxid, nor of intermediate stages in the oxidation of hemoglobin, hematin, or hemin. Neither has it been shown that these substances will yield an iron reaction short of their complete disintegration. These facts formed the basis upon which this investigation began.

The hydrogen peroxid employed was the perhydrol of Merck and Company (30 per cent. strength), and the dioxygen of the Oakland Chemical Company (3 per cent. strength), most of the work being done with the latter preparation. The stronger preparation seems to possess only two advantages, which are, rapidity of action and ability to oxidize some tissues, occasionally encountered, which show unusual resistance to oxidation. These advantages may be offset by the difficulty in controlling the action so as to obtain the degree of oxidation desired. Various dilutions of the strong peroxid can be used to good advantage.

Although hydrogen peroxid is unable to effect any appreciable degree of oxidation in unfixed red blood corpuscles, even after prolonged action, the change produced in fixed cells is quite rapid and pronounced. If a blood clot, fixed with alcohol, with formalin, or by boiling, be treated with 30 per cent. hydrogen peroxid, it rapidly changes color, becoming lighter brown and ultimately bleaching

to a perfectly colorless condition. The entire change requires but a few minutes, the actual time depending upon the size of the clot thus treated. The same changes are produced by weaker solutions of hydrogen peroxid, but require a much longer time. When such bleached clots are thoroughly washed with distilled water to remove the excess of the hydrogen peroxid and teased upon a slide, the fragments will give an instantaneous and intense Prussian blue reaction for iron, or the greenish black color with ammonium sulphid. If sections are prepared from oxidized clots—preferably fixed with formalin, which produces the best preservation of the red blood corpuscles—and treated with potassium ferrocyanid and dilute aqueous hydrochloric acid, the red cells will be found to give a definite, though light, blue reaction. With clots fixed with alcohol, the corpuscles show various degrees of laking and the reaction color is proportional to the amount of hemoglobin in the cells or concentrated at any part of the section. The fibrin and leucocytes also manifest the iron reaction.

Some interesting features in the development of such iron reactions in red cells can be traced by interrupting the oxidation at various stages and applying the tests for iron. Such experiments show that, with the Prussian blue method, the early stages of oxidation yield only a pale green color deepening to an olive green, greenish blue, and ultimately a clear blue, as the oxidation proceeds. These atypical color reactions are familiar to all who have had extensive experience with microchemical tests for iron, particularly in old specimens of hemosiderin, and we here find a possible explanation for such reactions. With the optimum degree of oxidation, the intensity of the reaction color is never so great in hemoglobin as is that exhibited by hemosiderin granules. This is undoubtedly due to the relatively greater amount of iron present in the latter substance.

Turning next to such iron-containing derivatives of hemoglobin as hemin and hematin, chemically pure products were prepared from rabbit's blood, according to the method of Schalfijew.[3] This hematin, when placed on a slide with a small amount of hydrogen peroxid (3 per cent.) and observed with a microscope, was found

[3] See Schäfer's Text Book of Physiology, Edinburgh and London, 1898, i, 252.

to undergo the same changes of color noted in blood clots. The dark brown or black granular masses change to lighter shades of brown and bleach from the periphery towards the center. Many of the smaller masses are completely decolorized within an hour. The freshly precipitated and still moist hematin is much more readily oxidized than a preparation which has been thoroughly dried. The decolorized hematin masses, though appearing to swell slightly, preserve their sharpness of outline and are highly refractile glass-like bodies. As oxidation proceeds, however, these masses swell decidedly, lose their sharpness of outline and gradually disintegrate. If the progress of the oxidation is interrupted below the complete destruction of the hematin, by draining off the excess of hydrogen peroxid and carefully drying over a flame or in the air, such preparations can be tested for iron, preferably by the Prussian blue method. All completely decolorized hematin and decolorized portions of hematin masses will exhibit an intense Prussian blue reaction, which appears immediately upon the addition of the reagents. The partially decolorized hematin shows a less pronounced reaction, depending upon the degree of decolorization (oxidation). Hematin granules that show beginning disintegration will give a diffuse precipitate of Prussian blue or a spreading halo about such granules.

With hemin and hydrogen peroxid, identically the same changes are produced, though hemin is oxidized with much greater difficulty, requiring approximately three to four hours to show many crystals which are decolorized, while some of the larger crystals will show patches of color even after twenty-four hours. These statements with reference to hemin do not agree with the findings of Gardner and Buckmaster,[4] who found hemin much more resistant even to the strongest hydrogen peroxid (perhydrol). They make no reference to the phenomena of decolorization, but describe the action as a corrosion of the hemin crystals. Some crystals do undoubtedly show this type of change which is probably more pronounced with the stronger solutions of hydrogen peroxid, which I have not employed in this instance. With carefully handled preparations and dilute peroxid, the process of decolorization can be readily

[4] *Jour. Physiol.*, 1906–7, xxxv, p. xxxii.

demonstrated. This decolorization takes place in the most peculiar forms, some of which are shown in the accompanying plate (figure 1). It is easy to see that what is really a partial decolorization of the hemin crystal might readily be mistaken for a corrosion, though with reduced illumination one would have no difficulty in distinguishing the decolorized portion of the crystal. Such decolorized crystals preserve their sharpness of outline, for a time at least, as is the case with hematin, though the tendency to disintegration seems stronger. Here again, the decolorized crystals and decolorized portions of crystals give a beautiful Prussian blue reaction for iron, as shown in figure 2.

If either paraffin or celloidin sections of tissues are treated with hydrogen peroxid, prior to the application of the iron reagents, the red blood corpuscles, laked hemoglobin, and iron-containing derivatives of hemoglobin behave essentially as above described. Certain differences, however, are to be noted. For reasons which thus far I have been unable to explain, tissue sections require a longer oxidation than is necessary with the isolated elements which I have studied. Tissues fixed with formalin are more slowly oxidized than those fixed with alcohol, and old tissues are more resistant to oxidation and react more feebly than freshly fixed tissues. Occasionally the reaction is entirely negative in tissues which have been kept for several years.

The behavior of hemosiderin, when treated with hydrogen peroxid, is so peculiar as to deserve especial mention. Although hemoglobin, hematin, and hemin are all relatively easily decolorized and, in the latter instances at least, disintegrated by 3 per cent. hydrogen peroxid, hemosiderin seems very slightly, if at all, affected after three to four days' action of the reagent, even when kept at a temperature of 37° C. Some change, however, has been produced, as is shown by its behavior towards iron reagents. First, alcoholic solutions of hydrochloric acid give only the faintest reaction, while aqueous solutions will produce a most intense reaction by the Prussian blue method. Again, those specimens of hemosiderin which previously have reacted imperfectly, or with difficulty, will give an instantaneous and typical iron reaction after treatment with hydrogen peroxid.

It is with some hesitancy that I speak of the reaction obtainable in other tissue elements, such as the various tissue cells, their nuclei, and cell granules, as no exhaustive study of such elements has been made. The tissues which were studied by the peroxid method included liver, kidney, spleen, lymph nodes, and a few specimens each of chronic inflammatory tissues, striated muscle, and carcinomata. As a rule, the cell body in these tissues reacted very faintly for iron, if at all. The reaction was variable in all cases. Some cells, such as liver parenchyma, however, seemed more constant than others and frequently exhibited a well marked iron reaction. The same statements apply with equal force to the nuclei of these cells. While in some instances the nuclear reaction was intense, in others, it was very faint. In a few instances I have failed entirely to obtain anything which I could recognize as a positive iron reaction, particularly with some very old specimens. As to cell granules, I have studied only the eosinophil granules of cells found in chronic inflammatory and malignant conditions about the genito-urinary tract of the human subject, and the eosinophil granules in chronic inflammatory tissues from the horse. The granules from the latter source are extremely large and furnish excellent material for study. While with the human tissues I was not able, in all instances, to detect a positive iron reaction in the eosinophil granules, I found that the eosinophil granules of the horse react uniformly and sharply.

When it is stated that all the above tissue elements react for iron, as has been indicated, perhaps all has been said that can be said with certainty. However, there must still remain an element of doubt as to the significance of such reactions. The results reported by those workers who have employed acid alcohols, or ammonium sulphid and glycerin for prolonged intervals, or at high temperatures, to obtain microchemical reactions for iron in the chromatin of cell nuclei, are certainly not absolutely conclusive. The possibility of an ante- or post-mortem absorption of hemoglobin by these structures can not be entirely excluded. Further than this, the acid alcohols tend to dissolve any inorganic or albuminate iron that may be present, as well as to extract hematin from the red blood corpuscles. In view of the absorptive power of nuclear material for

iron compounds, the use of acid alcohols is certainly open to question.

While the trace of acid present in most preparations of hydrogen peroxid still leaves some legitimate ground for questioning the results obtained in some cases, still I believe the method is, on the whole, more reliable than other methods. Yet, at present, I am not at all prepared to claim more than the production of a positive iron reaction in the elements mentioned. In fact, the most pronounced nuclear reactions have been obtained in just those instances where the possibility of the absorption of hemoglobin by such nuclei seemed highly probable. As to the iron reaction in eosinophil granules, this was described by Barker[5] some years ago, the results being obtained by the prolonged use of warm ammonium sulphid and glycerin. Although I have seen no confirmation of this work, there can be no doubt as to the ability to obtain an iron reaction from the eosinophil granules of the horse, at least. The source or significance of this iron is another question and, in the tissues I have studied, might possibly be referable to hemoglobin absorbed from the erythrocytes of the inflammatory tissues. In this case, however, it must needs be modified by or united to the cell granules in some way. The best means I have found for determining the presence of dissolved hemoglobin in all these tissue elements is that used by Browicz.[6] According to Browicz, hemoglobin in solution, when fixed with formalin, is precipitated as brown or black granular or crystalline material and all structures containing dissolved hemoglobin show the presence of this precipitate. If this test is wholly reliable, and thus far I have found no reason to question it, tissue cells, cell nuclei, and eosinophil granules contain varying amounts of iron, not behaving as dissolved hemoglobin, which are demonstrable after an appropriate oxidation with hydrogen peroxid.

The technique for demonstrating iron in the various structures, as described above, must be varied to suit the demands of the particular case. Most workers prefer alcohol as a fixative in the study of iron in tissues, as it is claimed that the largest amount of demon-

[5] *Bull. Johns Hopkins Hosp.*, 1894, v, 93.
[6] *Virchows Arch. f. path. Anat.*, 1900, clxii, 373.

strable iron is obtained after this method of fixation. The method of fixation which shows the largest amount of iron, however, does not necessarily represent the true picture. This I am sure is, at times, the case with alcohol, which produces laking of corpuscles and diffusion of hemoglobin and may permit of post-mortem changes in such diffused hemoglobin. For these reasons, formalin is to be preferred for purposes of fixation in some cases. Except where very thin sections are to be used, celloidin sections are usually more satisfactory than paraffin sections. As to the proper length of time that sections should be oxidized with hydrogen peroxid no absolute statement can be made. The degree of oxidation necessary for the demonstration of iron in any particular element will vary slightly with different tissues, and there is a marked difference in the length of time necessary to render demonstrable the iron of such bodies as red blood corpuscles and that of cell nuclei.

The optimum time with 3 per cent. hydrogen peroxid will usually be found to be about as follows: hemoglobin and its modifications (not including hemosiderin) require twelve to twenty-four hours; eosinophil granules require twenty-four to thirty-six hours; cell nuclei and protoplasm require thirty-six to forty-eight hours, though frequently seventy-two hours will be found to give still better results.

Wherever possible, the dilute solutions of hydrogen peroxid should be employed in preference to the strong, for the reasons previously noted. The treatment with hydrogen peroxid should be followed by thorough washing with distilled water. The application of the actual tests for iron may be carried out according to individual preference, except that alcoholic solutions of hydrochloric acid seem not to yield as good results as the aqueous solutions.

In addition to the experiments with hydrogen peroxid, which have been described in some detail, potassium bichromate, chromic acid, potassium permanganate, and potassium chlorate, in acid solutions, have been used as oxidizing reagents in the same manner as the peroxid. There are obvious objections to the use of such compounds. The chromium compounds might possibly give rise to confusing results unless completely removed from the section; and this is not readily accomplished. Likewise the necessity of using

FIG. 1.

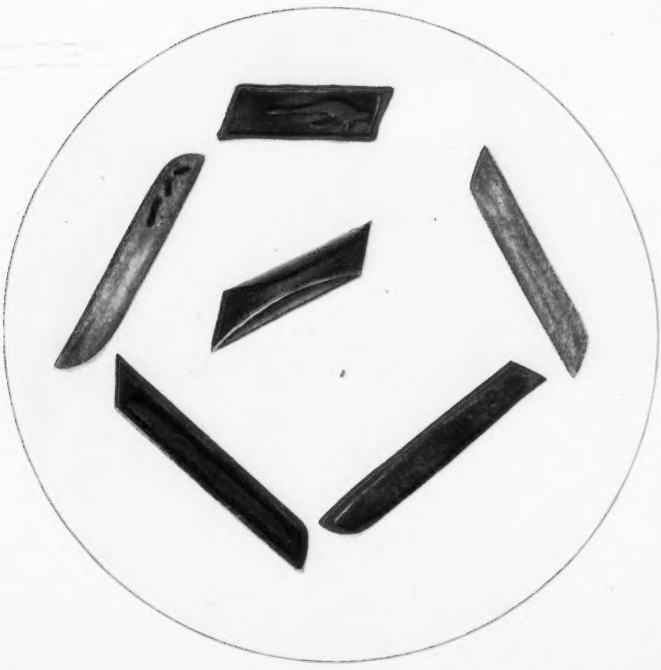

FIG. 2.

such substances as oxalic acid to decolorize the section after the use of the permanganate may result in the removal of much of the iron that has been liberated. Finally, the acid in the solution constantly tends to extract the iron as it is oxidized. Notwithstanding these objections, very good results have followed their use in some instances, although I see nothing to commend their use in preference to the peroxid of hydrogen. They are mentioned in this connection as means of confirming the results previously described and as furnishing additional evidence to the value of the principle of oxidation in the microchemical detection of iron.

SUMMARY.

1. It has been shown that hemoglobin in solution or within the red blood corpuscles can be oxidized by hydrogen peroxid to form a colorless substance capable of manifesting a microchemical reaction for iron, the type of the reaction depending upon the degree of oxidation.

2. It has been further shown that hematin and hemin, when treated with hydrogen peroxid, are decolorized. The colorless or nearly colorless body, preserving the form of the original body and being an intermediate product of its oxidation, will also exhibit a typical reaction for iron.

3. A method has been described for obtaining microchemical reaction for iron from hemoglobin and its various modifications and iron-containing derivatives, from tissue cells, from cell nuclei, and from eosinophil granules, by an appropriate degree of oxidation with hydrogen peroxid.

EXPLANATION OF PLATE LXII.

FIG. 1. Hemin crystals showing various types and stages of their oxidation by hydrogen peroxid.

FIG. 2. Similar crystals showing the Prussian blue reaction for iron.

VACCINE TREATMENT IN THE PREVENTION OF DYSENTERY IN INFANTS.*

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This work was undertaken during the summer of 1910 in order to find out, if possible, whether vaccine treatment would have any effect on either the morbidity or the mortality of the dysentery of infants. The months chosen for this work were July, August, and the early part of September. During these months, by far the greatest number of cases of infantile dysentery occur in this country. The difficulties of controlling such cases in giving the vaccine treatment and in getting the specimens for bacteriological examinations can easily be understood.

All the cases vaccinated were under two years of age and clinically showed no signs of dysentery or of any very acute gastro-intestinal disorders. The vaccine was not administered to any child having a temperature above 38° C. In as many cases as possible specimens of the stools were obtained from the treated cases for bacteriological examination and an equal number of control cases were followed bacteriologically and clinically. In this way it was hoped that some conclusion could be reached which would represent the value of this work carried out under the prevailing conditions.

Historical.—The history of preventive vaccination against dysentery dates back to 1898, when Shiga (1) tried the subcutaneous injection of small amounts of killed cultures of the *B. dysenteriæ* (Shiga). This method produced such marked general and local reactions that he abandoned it in favor of a combination of killed cultures and a specific serum injected simultaneously. From 1898 to 1900 (2) he vaccinated 10,000 Japanese in this way. These first injections were followed three or four days later by a dose of vaccine twice as large, but with no specific serum. His results at this time showed no diminu-

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tion in the morbidity. From 30 to 40 per cent. the mortality diminished to almost nothing. The immunity secured was of very short duration, lasting only three or four weeks.

Lüdke (3), in 1905, published an experimental study on the production of agglutinins and antibodies in rabbits and on the power of the serum so produced to kill the dysentery bacillus. Both Shiga and Lüdke showed that after using the Shiga dysentery bacillus the general and local reactions were very marked and at times quite dangerous. In 1909, Ch. Dopter (4) published a complete study of the preventive vaccination by the dysentery bacillus. In conjunction with Vaillard (5), in 1903, he had shown that adult mice or white rats were the best laboratory animals for experimentation with this organism. He therefore worked with adult mice weighing twenty grams, giving them .00001 of a gram of a killed dried culture of dysentery bacilli. His results showed that with mice such vaccination in 40 to 50 per cent. of cases can confer an immunity against a fatal dose and that such an immunity appears about twelve days after the first injection and lasts from four to six weeks. During the negative phase, i. e. while the animal is acquiring its immunity, it is more susceptible to a fatal dose than the control. The local and general reactions of such vaccines were quite marked. It is of great importance to note that there is a negative phase, for this fact makes it impractical to use this method when there is an epidemic of dysentery, or when the disease is very prevalent, as it is during the summer months. Dopter cites the use of this method in several human cases. For instance, Kruse gave himself a subcutaneous injection of one cubic centimeter of a killed culture. At the site of inoculation there was marked swelling, edema, and pain. After about four days, during which time there was slight abatement of the local reaction, the general symptoms of fever and prostration appeared. Shiga injected into his forearm a small dose of a killed culture. In a few hours his temperature rose to 38.6°, and there was marked swelling, edema, and pain at the site of inoculation. His axillary glands also became swollen. After about three days, during which time the symptoms abated slightly, the swelling and temperature recurred. Rosenthal vaccinated himself and his laboratory boy, inoculating one cubic centimeter of a killed culture subcutaneously. There was a marked local reaction, as well as general constitutional effect, headache, and arthritis. These personal experiences show the discomfort of such a method of vaccination. The same results were obtained by Dopter by using the autolytic products of the dysentery bacillus (Shiga). The immunity did not last longer than six weeks and took from ten to twelve days to appear. The negative phase was also present.

Shiga used the antidysentery serum to produce a passive immunity. The duration of such an immunity is very short, not exceeding ten days. This was shown by Kruse in 1904 when he vaccinated ten people, each with two cubic centimeters of antiserum. Only one of this number took the disease, the first symptoms in this case occurring three days after the injection of serum. This amount, he concluded, was too small. In 1907, Lallemant used ten cubic centimeter doses of antidysenteric serum in treating sixty cases at the insane asylum at Quatre-Mares. None of this number became sick during the epidemic. Rosculet (6) tried this method in quite a large epidemic of dysentery by giving five cubic centimeters of an antidysenteric serum to each of eighteen people.

In none of this number did the disease develop, although all were exposed; while of eighteen other people who were not vaccinated, fourteen contracted the disease. Michiels (7), during an epidemic at Chauvigny, gave an injection of ten cubic centimeters to each of fifteen people. Only one of this number contracted the disease, the first symptoms appearing in this instance nine days after the injection. It would appear from this case, and from Michiels's own personal experience, that the duration of this passive immunity does not exceed ten days.

Mixed Vaccination with Antiserum and Bacterial Vaccine.—In August, 1900, dysentery broke out in a Japanese village. During one month there were twenty-eight cases. Shiga (8) inoculated all the inhabitants over four years of age that were not already infected. The first injection consisted of antiserum and bacterial vaccine in equal amounts, and four or five days later he gave a mixture of eighty parts of bacterial vaccine to twenty of antiserum. All these injections were followed by mild general and local reactions. In this village epidemic, only two cases appeared after these inoculations.

Dopter tried these same experiments on mice. He concludes from this study that vaccination with antiserum and bacterial vaccine gives a speedy immunity which is practically immediate in the great majority of cases. Immunity, however, lasts only about four weeks. This method dispenses with the negative phase and therefore prevents the subject from being more susceptible to an infection with the dysentery bacillus. The local and general reactions from this method are also markedly less than when the bacterial vaccine is used alone. Dopter concurred with Beinarowitsch in believing that the quantity of serum given affects the duration of the immunity in an inverse ratio, the smaller the quantity of antiserum given in combination with the bacterial vaccine, the longer the immunity.

Dopter tried a new plan for producing immunity, using sensitized dysentery bacilli for vaccination. The vaccine was made up as follows: Shiga dysentery bacilli (killed by being heated at 60° C. for an hour and dried in a vacuum) were made into an emulsion with physiological salt solution. To this emulsion was added antidysenteric serum of a very high agglutinating power. This was allowed to stand at room temperature for about twelve hours. By this time the bacilli were strongly agglutinated, had become sensitized, and had fallen to the bottom of the tube. The supernatant fluid which was clear was decanted. The precipitate was washed and centrifugalized twice in physiological salt solution and the last sediment was made into an emulsion with physiological salt solution.

Dopter carried out a long series of experiments with mice, using this sensitized vaccine, and he concludes (1) that mice vaccinated by sensitized bacilli acquire an immunity in about four days; and (2) that there is no negative phase —the vaccinated animal being no more susceptible than the control. The immunity persists at least four and one half months. There are scarcely any local or general reactions from this sensitized vaccine.

These are practically the same conclusions as those arrived at by Besredka (9) who used sensitized cultures in studying the cholera vibrio and the plague bacillus. The only possible objection to this means of producing immunity is that the immunity does not appear for four days, although there is no negative

phase during this period. The sensitized vaccine method just described and the method of administering antiserum in combination with bacterial vaccine, seem to be the two methods of choice.

For this special investigation the latter method was chosen for the following reasons: (1) It had been tried on human cases, apparently with good results, whereas the first method, which, however, seems more likely to produce the best results, had not been tried on human cases. However, an experimental study of this method is being undertaken and we hope to be able to say something further concerning it as a means of preventive vaccination. (2) A previous study (10) had shown that the main cause of infantile dysentery is the organism of Flexner and not that of Shiga, which bacillus has been used in all the previously noted experiments. During this study we found, as have also other investigators, that the Flexner dysentery bacillus is far less toxic to the ordinary laboratory animal than is the Shiga type of *B. dysenteriæ*. This we learned in trying to produce an antiserum of high potency in rabbits. Although we were fairly successful in producing an antiserum to the Flexner type of the *B. dysenteriæ*, it was extremely difficult to do so with the Shiga type. Further, we found, as other investigators have done, that the Flexner organism may be present in the intestinal tract without causing the characteristic clinical symptoms of acute dysentery, thus showing that even in the human system this type of dysentery bacillus can exist without producing marked toxic symptoms. It seemed fair, then, to conclude that a vaccine made from an organism isolated from a case of infantile dysentery (Flexner), would be the most appropriate one to use as a preventive. The following plan was therefore adopted. A standard vaccine was made from a twenty-four hour agar growth of *B. dysenteriæ* (Flexner) of such strength that one cubic centimeter of the emulsion contained one hundred million bacilli. At the first injection, one cubic centimeter of an antidysenteric serum was used in combination with fifty million bacilli, or one half cubic centimeter of the standard emulsion.

The plan was to make three injections, the second to be given five days after the first. This was not always possible, however, from the character of the clinic. The majority of the patients

received only one or two injections and the interval separating the first from the second inoculation varied from five days to three weeks. Fifty-one patients received one injection, and forty-four received two or three. In all, ninety-five patients were vaccinated.

The manifestations following the vaccinations varied from a slight local reaction and a temperature of 99° F., with slight fussiness, to a very marked local swelling, edema, and considerable tenderness. In no case was there any abscess formation, and usually the most marked reactions disappeared within twenty-four hours. All the vaccinations were given in the morning, and each patient was visited from four to six hours later, when the local condition and temperature were noted. The highest temperature noted was 103°. When the temperature was over 101° the patient was always visited the following morning, and in no instance was the temperature above 99° on this second visit.

From thirty-three of these vaccinated patients, one to four fecal cultures were taken by the method used by Kendall (11).

In this is employed a small glass tube with rounded ends, one of which is plugged with cotton. The tube is placed within an ordinary thick-walled culture tube and the whole is sterilized. The cultures were obtained by passing the sterile tubes into the rectum, and at the end of the clinic these tubes were expressed to the laboratory where the contents of the tube were discharged into a tube of plain broth. From this emulsion, the isolation of the *B. dysenteriæ* was carried out by a modification of the method used by Kendall and Walker. Using varying amounts of the emulsion according to the character of the stool and the strength of the emulsion, two large plates were made on Endo's medium and incubated for eighteen hours. On these plates *B. dysenteriæ* appear as slightly elevated, clear, colorless colonies measuring in diameter from .5 to 1.5 millimeters, according to the total number of colonies on the plate.

All suspicious colonies were fished into litmus mannite semisolid media and incubated for eighteen to twenty-four hours. If at this time there was growth characteristic of the Flexner or Shiga type of *B. dysenteriæ,* a tube of plain broth (reaction —.5) and a tube of litmus milk were inoculated with the culture and incubated for a day. If at the end of this time the litmus milk showed the characteristic lilac color, microscopic agglutination of the broth culture at a dilution of 1 to 200 was tried.

Later in these investigations, in order to make a diagnosis more quickly, since a large number of specimens were being studied, suspicious colonies from the plates were fished directly into plain broth (made from meat extract, reaction —.5). This was incubated for about eighteen hours and then agglutinating horse serum was added to the tube. A drop of Flexner serum was used first, and then, if in three or four hours there was no agglutination, a drop of Shiga

antidysenteric serum was added. If, after either, agglutination was positive, the supernatant broth was drawn off and sterile bouillon was added to the tube. The tube was shaken, a loopful transferred to another tube of broth, and plates made from this. After incubation, the colonies were fished and carried through mannite semisolid litmus milk and the agglutination test made a second time. The strains were finally transferred to agar slants for preservation. By this method a tentative diagnosis was reached in about thirty-six hours. It was found that a positive microscopic agglutination could be allowed to stand for several hours and the agglutinated organisms would grow when transferred to fresh broth.

Of the thirty-six cases studied culturally, six proved already to have the dysentery organism. Five of them had the Flexner type of the dysentery organism, and one the Shiga.

Two of these patients received three vaccinations, as follows: the first vaccination consisted of one cubic centimeter of antiserum and one half cubic centimeter of the standard emulsion (fifty million bacilli); on the second inoculation, they received one half cubic centimeter of the standard emulsion and no antiserum; and on the third inoculation, one cubic centimeter of the standard emulsion. Three of the patients received only two injections, the first with antiserum and the second without. One patient received only one injection of antiserum and vaccine.

The immediate reaction in all these cases was very mild, the highest temperature being 101.5°. All the local reactions were of the mildest type and there was no general reaction noted beyond slight fussiness. In no case was the immediate intestinal condition aggravated, and in all but one case no further intestinal trouble has appeared up to the present.

One patient receiving two doses of vaccine, the last being given on August 7, did well until an acute intestinal infection developed on September 8. This child was entered in one of the hospitals as having an acute infectious diarrhea. This attack was only of moderate severity, and the child when seen last, October 24, was in the best condition.

In none of these six patients was the dysentery organism suspected before the vaccine was given, as it was the intention at first to avoid giving vaccine treatment to any patient having dysentery infection.

The results, however, in these six cases show very clearly that vaccine made from the *B. dysenteriæ* (Flexner) is not contra-

indicated in those cases which may have this organism in a clinically unrecognizable form.

Out of the ninety-five patients vaccinated, there were two deaths. In the two cases that ended fatally, the original cultures were negative for the *B. dysenteriæ*. Each patient received two vaccinations. One child contracted acute infectious diarrhea seventeen days after its last inoculation and died ten days later. The outcome is interesting from the standpoint of hygiene. The family lived under conditions of the worst kind, and with flies in superabundance. Undoubtedly the features which, more than any others, contributed to the fatal result in this case, were the utter lack of intelligence on the part of the family and their indifference to the sickness of the child. With these difficulties it was impossible to cope.

The second case was that of a very delicate child who lived under the worst sanitary conditions of filthy people. Here also the flies were numerous. This child did well for one month after its last inoculation, when it contracted acute infectious diarrhea and died two weeks later.

The other ninety-three cases did perfectly well throughout the whole summer and when last seen, sometime in the latter part of October, were in the best condition.

It is interesting to compare these cases with ninety-seven which did not receive any vaccination and which proved culturally to be negative as far as the presence of the *B. dysenteriæ* was concerned. Of this number, three died, apparently of acute infectious diarrhea, after illnesses of from three to five days. No bacteriological examinations were made on these cases and autopsies could not be obtained.

In twenty-five cases studied culturally but not vaccinated, *B. dysenteriæ* were found. In twenty-four of these the Flexner type of the organism was present, and in only one, that of Shiga. There has been one death from this group of twenty-five cases. In only one or two of these cases was the presence of the *B. dysenteriæ* suspected at the time the culture was taken, and in the case that died, the final acute attack was very short and severe. This is of interest when compared with the subacute form of attack in the three cases that developed acute infectious diarrhea later in the summer.

Agglutination.—The agglutination test was applied in thirty-six of the ninety-five cases vaccinated. Agglutination was negative at 1 to 50 in some, and positive at 1 to 400 in several of the vaccinated cases. No difference was noted in the agglutinating power of the six cases in which the dysentery bacillus was isolated. A few control agglutinations were obtained from some of the cases in which the dysentery bacillus had been isolated by culture, but which were not given the vaccine. One of these cases agglutinated very strongly up to 1 to 100, another only mildly at 1 to 100, and a third only mildly at 1 to 50. Several control cases which were culturally negative gave agglutination reactions. One patient who was not vaccinated, from whom the organisms were not cultivated, and who clinically was not suffering from infectious diarrhea, gave a strong agglutination at 1 to 100.

The home conditions of all the vaccinated patients were studied and recorded. There can be no question that whatever the treatment outlined at a clinic may be, or whatever preventive measures are instituted, these are of little or no avail in the unsanitary conditions under which a fair majority of these children were compelled to exist.

The question of the presence of flies appeared to be a most important one, and toward the end of the summer an attempt was made to discover how far the fly entered into the carrying of the dysentery organism. To this end sterilized fly-traps were set in the homes of several patients from whom the dysentery bacilli had been recovered. In two out of three such attempts, the dysentery organism was recovered from an emulsion made from these flies. The manner of procedure was as follows:—

An ordinary wire fly-trap, sterilized by heat, was left at the house for two or three days. The traps were brought to the laboratory and were set in a cold room (4° C.) for fifteen minutes. The cold numbed the flies to such an extent that they could be picked out of the cage with sterile forceps and placed in broth. The drowned flies were ground in a sterile mortar and from the emulsion plates were made, some of them being on Endo's medium. Any suspicious colonies were run through the ordinary media for isolating the dysentery organism, and finally treated for agglutination. In the two cases in which the Flexner organism had been isolated, this

same organism was also recovered from the flies. It is interesting to note that the *B. dysenteriæ* are carried by the ordinary house-fly and that these flies may be one of the causes of spreading this infection during the months when they are so abundant.

CONCLUSIONS.

This investigation is very limited and the number of patients treated is few, yet the following conclusions may not be amiss:—

1. The vaccine in no instance did harm, and the reactions in the majority were very mild and of short duration.

2. Cases in which bacteriologically *B. dysenteriæ* were proved to be present, but in a clinically unrecognizable form, were not affected differently from those which were culturally negative. This may in part be due to the fact that this mode of vaccination produces an immediate passive immunity, while the bacterial vaccine is producing an active immunity.

3. The work is of interest and holds out a possible means of preventing the great mortality from infantile dysentery during the summer months.

We wish to express our indebtedness to Dr. John Lovett Morse, who rendered this research possible by raising a fund sufficient to carry it on, and to Professor Milton J. Rosenau, for his supervision and suggestions. We wish also to thank Dr. Grace Atkins Jordan for valuable assistance in getting specimens and for clinical observations in many of these cases. Finally, we desire to express our indebtedness to the undergraduate students who assisted in many ways.

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CHARACTERISTICS OF GROWTH OF SARCOMA AND CARCINOMA CULTIVATED IN VITRO.*

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PLATES LXIII–LXXII.

In this article we shall describe the growth of rat and mouse sarcomata and of mouse carcinomata when cultivated *in vitro*. By growth we mean the performance of metabolic functions by the cells and cell multiplication.

Historical.—The essentials of the method of cultivating tissues *in vitro* we owe to R. G. Harrison (1907, 1910), who used lymph obtained from the dorsal lymph sacs of frogs as a medium for the cultivation of the tissues of frog embryos, and especially as a means of stuyding the outgrowth of nerve fibres from nerve cells. The successful work of Harrison is too well known to require detailed mention. M. T. Burrows (1910, 1911), working in Professor Harrison's laboratory at Yale University, greatly improved Harrison's original method by perfecting a technique whereby the tissues of a warm-blooded animal could be cultivated outside the body in the plasma of an animal of the same species. The modifications introduced by Burrows are scarcely second in importance and value to Harrison's original conception.

Several observers (Ljunggren, 1898, Jolly, 1903, Beebe and Ewing, 1906, Carrel, 1910, Volpino, 1911) have shown that tissues from warm-blooded animals may be kept alive for a time by employing as media blood serum, defibrinated blood, ascitic fluid, etc. Carrel and Burrows (1911) point out with justice, we think, the great difference that exists between methods which simply permit the temporary survival of cells outside the body and a method which insures the growth of such cells.

The technique employed by Leo Loeb (1902) in studying the growth of cells in foreign media occupies a position midway between procedures which permit merely the temporary survival of cells outside the body and the method of cultivation of tissues *in vitro*. In a study of the regeneration of epithelium in wounds of the skin, Loeb observed that the growing epithelium penetrated the scab covering the wound, and frequently surrounded in its growth small masses of the scab. This demonstration of the possibility of isolated and independent growth on the part of the epithelium of the skin led him to test the viability and power of growth of pieces of guinea pig skin embedded in small blocks of

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coagulated blood serum and agar, kept within the body of the animal. A second very ingenious method used by Loeb consisted in bringing a small undetached flap of the skin of a guinea pig's ear into contact with a piece of agar or coagulated blood-serum embedded in the deeper parts of the ear. By each of these methods Loeb was able to demonstrate that epithelium under such conditions remained alive, penetrated the foreign media, and proliferated. The many interesting details of Loeb's work must be read in his own papers; we wish simply to point out that his experimental methods, considered in their essentials, approximate very closely those of Harrison and Burrows. The defects and limitations of Loeb's methods are obvious; his procedures are in no way comparable with the refined and certain methods elaborated by Harrison and Burrows.

Burrows (1910) studied in Professor Harrison's laboratory the outgrowth of nerve fibers from nerve cells, using the tissues of chick embryos and plasma obtained from adult chickens. He also observed the growth, including ameboid movements and karyokinetic multiplication of mesenchymal tissues. He has recorded in a recent paper (1911) many important observations upon the growing tissues of chick embryos.

Carrel and Burrows (1911) in a series of notes have reported details of the growth of many adult tissues of warm-blooded animals.

Lambert and Hanes (1911) have reported briefly the results of studies upon the growth in vitro of the transplantable sarcomata and carcinomata of rats and mice. In this and the following paper the details of these studies will be presented.

Technique.—In cultivating rat and mouse tumors, we have employed the method of Harrison (1910), as modified and improved by Burrows (1911), with certain minor adaptations rendered necessary by the small size of the animals used. The diminutiveness and delicacy of the carotid artery in mice and young rats offers the chief difficulty in obtaining blood from these animals. The use of a cannula is impracticable. We have employed successfully the following technique: The carotid artery is exposed, cleaned, and ligated distally. A *serre fine* clamp is placed on the artery as far proximally as possible, and with sharp scissors the wall of the vessel is nicked near the distal ligature. The wall of the artery is picked up with fine pointed forceps, the vessel is divided distally, the *serre fine* clamp is removed and the blood allowed to flow into heavy paraffin-coated glass tubes, great care being taken to prevent contamination with tissue juices (activator). The separation of the plasma from the formed elements of the blood and the subsequent preparation of the cultures have recently been described in detail by Carrel and Burrows (1911).

CHARACTERISTICS OF GROWTH IN VITRO OF SARCOMA.

Rat and mouse sarcomata cultivated *in vitro* do not differ essentially in their manner of growth. They conform to a type of growth that would seem to be characteristic of tissues of mesenchymal origin.

If a culture of sarcoma freshly prepared *in vitro* be examined under the microscope, the edges of the piece of tissue are seen to be sharply defined and the tissue is uniformly dense and opaque. Six to twelve hours later the edges of the tissue are thickly beset with projecting cells (figure 1), and many isolated cells with extremely irregular outlines are seen to have passed out into the surrounding plasma. The tissue itself gives the impression of being less compact and more translucent than formerly. The number of cells in the plasma now increases rapidly from hour to hour (figure 2), and concomitantly the original mass of tissue becomes progressively looser in texture, and its edges pass imperceptibly into radial strands of cells lying either in contact with one another or perfectly isolated. The original mass of tissue may become resolved completely into its component cells which wander into the plasma. Serial sections (figure 3) of preparations at this stage of growth confirm very beautifully the details observed in the living cultures.

The passage of cells into the surrounding plasma, and the diminution in bulk and density of the original piece of tissue, constitute the phenomena of growth as observed under the low powers of the microscope. Seen with the unaided eye, growing preparations exhibit a filmy halo surrounding the original mass of tissue. For a closer study of the exact mechanism whereby the cells move into the surrounding plasma, and for the observance of the more intimate structure of the cells, higher powers must be used, and it is necessary to supplement the study of the living tissue by fixed and stained preparations of whole cultures and by serial sections.

The cells which have passed into the plasma, examined with the high power (dry) of the microscope, present two fairly distinct morphological types. One type is long and spindle-shaped (figure 4) with the cytoplasm forming thread-like prolongations from each end, which extend for great distances into the plasma. These attenuated processes usually terminate in several small prickles.

The nucleus lies in the middle of the cell, and is ovoid in outline or distinctly fusiform. The second type of cell, which in actively growing cultures greatly predominates, is characterized by the extreme irregularity of its shape, due to pseudopods which project from the cell in the most bizarre manner (figure 5). If one observes such cells for a time, the pseudopods are seen to undergo alterations in shape. Each pseudopod terminates in several thread-like processes, and these are thrown out and retracted from time to time. Now and then one of these delicate processes enlarges and becomes rounded as the cytoplasm streams into it. By this mechanism the cell alters its spacial relations, wandering further and further from the original tissue. Cells showing active ameboid movements assume a compact spherical form if allowed to cool.

We are inclined to regard these two types of cells as merely morphological variations, and not generically separate types. It is impossible to tell in a growing culture of sarcoma which cells were originally stroma cells and which were sarcomatous. Since, however, all the cells of the implanted tissue may show the phenomena of growth, the question is one of secondary importance. Both stroma and sarcoma cells are of mesenchymal origin, and both probably conform to the same type of growth.

Typical karyokinetic figures are not uncommon in the growing sarcoma cells, though observable only in stained preparations. Cells with two nuclei are frequently seen.

We have emphasized the ameboid wandering of the cells into the plasma. The cells lying compactly massed in the original piece of tissue, with the normal vascular food supply interrupted, are most unfavorably situated for obtaining nutriment. It is in response to the demands for food, we think, that the cells migrate into the plasma, whose fibrin meshwork is filled with an abundance of nutrient material. If this interpretation of the cell migration is correct, the cytoplasm of the cells might conceivably show recognizable signs of active metabolism. This seems to be the case. The cytoplasm of migrating cells (figure 4) exhibits very early small highly refractile droplets, which stain brilliantly with specific fat stains, such as Sudan III and Scharlach R., and otherwise respond to all the tests for neutral fats. These fat droplets increase in num-

ber with the age of the cell, until the cytoplasm is often literally packed with them. They show little tendency to fuse into large droplets, and flow with the cytoplasm into newly formed pseudopods, recalling the passive movements of foreign inclusions in amebae.

We do not, for a moment, regard these granules of fat as an evidence of degeneration, as has been suggested; they are rather the strongest proof, we think, of active anabolic function on the part of the cell. They are synthesized by the cell from the soluble elements of neutral fat present in the plasma; fat granules can not be demonstrated in the plasma itself. Cells containing numerous fat granules show active ameboid movements (figure 6) and undergo karyokinetic division. These are evidences of vitality which do not harmonize with the view that the fat is a result of "fatty degeneration" of the cytoplasm. The accumulation of fat in the cells indicates an impairment of katabolic activity through influences which are easily understood. Far from being strange or new, this process of fat accumulation, or infiltration, is seen frequently throughout the cells of the body.

In another place (1911a), we have pointed out that the power of independent ameboid movement shown by growing tumor cells offers a very reasonable explanation of the infiltrative growth of malignant tumors. The cells of a rapidly growing tumor are soon placed in a position analogous, from the standpoint of food supply, to that of a mass of tumor cells embedded in plasma. The nutritional demands of the cells may be regarded as a stimulus which results in an invasion by ameboid movement of surrounding tissues, lymph-spaces, lymph-vessels, and blood-vessels.

When suitable preparations of growing sarcoma cells are properly fixed and stained, another type of granule is seen (figure 6). These are the cell granules of Altmann. We can not enter at this time into a detailed discussion of these granules, since a proper presentation of the subject would necessitate a review of the rather specialized literature dealing with the granular structure of protoplasm. The reader whose interest leads him in this direction will find in the first volume of Martin Heidenhain's recent work, "Plasma und Zelle" a full critique of the subject. We accord to these gran-

ules more than passing mention because Beckton (1909) has stated that their absence from tumor cells is a characteristic of malignancy. Bensley (1910) has shown that Beckton is wrong with regard to human malignant tumors; the cells do contain Altmann's granules, and even in increased numbers as compared with the normal cells of the tissue from which the tumor arose. Using Altmann's fixative and stain, we have demonstrated abundant protoplasmic granules in the cells of mouse and rat sarcomata, both when growing in the animal and when cultivated *in vitro*. Furthermore, we have been able to demonstrate these granules in the living cell by a method of intra-vital staining which will now be described.

If finely powdered carmin be added to the uncoagulated plasma used for cultivating sarcoma cells, these cells take up the granules in a very actively phagocytic manner (figure 7). The carmin is partially dissolved within the cell, and the cell granules are thereby stained. This process takes place in living cells showing ameboid movements and phagocytic activity, so we can not doubt that here we have a vital demonstration of the cell granules which are also demonstrable by special methods of fixing and staining.

There is a variation in the manner of growth *in vitro* of sarcomatous tissue, which occurs so frequently and presents such well-defined characteristics that it demands description. This is the ring form of growth (figure 8). In this type, the growing cells do not spread uniformly in all directions from the central mass of tissue, but the plasma retracts from the tissue in an ovoid or circular fashion, leaving the tissue situated at one point of the circumference, like the setting in a signet ring. The growing cells are found to have spread out in a one-celled layer in a thin film of plasma which remains adherent to the cover glass after the retraction of the plasma, and also to have massed themselves in the edge of the plasma ring. From this secondary deposit of cells, ameboid wandering takes place into the plasma. If the remainder of the original piece of tissue be removed, and the space bounded by the ring-like deposit of cells be filled with fresh plasma, cells wander into this new supply of medium from the inner surface of the ring. Similar ring-like growths of mesenchymal tissue have been described by Harrison (1910) and Burrows (1911). Such a form of growth

might be explained by the action of a proteolytic ferment on the fibrin of the plasma, allowing the contracting plasma to retract from the embedded tissue. This ring form of growth is almost the rule when mouse sarcoma is cultivated in rat plasma. We have found the central, one-celled layer of growth exquisitely adapted to finer cytological studies.

CHARACTERISTICS OF GROWTH IN VITRO OF MOUSE CARCINOMA.

Mouse carcinoma cultivated under the same conditions as sarcoma, grows with equal readiness, although the details of growth are very different. After twenty-four hours of growth *in vitro* a culture of carcinoma presents the following appearance: The original piece of tissue has become very much thinner and more translucent, due to a spreading out of the cells on all sides. The cells, however, do not migrate into the plasma separately, as do sarcoma cells, but move in a continuous sheet or layer, which is only one cell in thickness (figure 9). The advancing edge of the growth is composed of a very delicate sheet of cytoplasm, which shows ameboid changes of form with numerous pseudopods (figure 10). The boundaries of the cells composing the sheet of growing carcinoma can not be seen with clearness, even in stained preparations. There is a heaping up of the cells just behind the advancing hyaline fringe of protoplasm (figure 9). As the cells grow out from the original mass, their nuclei increase in area, as does the cytoplasm, so that they present the picture of very large nuclei lying rather widely separated by cytoplasm.

Growing carcinoma cells, like sarcoma cells, fill themselves rather rapidly with fat granules. They also take up phagocytically carmin granules placed in the plasma, as do sarcoma cells.

Karyokinetic figures (figure 10) are frequently observed; we have seen them in preparations as old as five days.

Carcinomata are composed of epithelial cells lying in a stroma of connective tissue. We have pointed out that these two varieties of tissue vary characteristically in their manner of growth *in vitro*. It should therefore be possible to distinguish without difficulty the growth of stroma from that of carcinoma. Some of our preparations show very clearly a mixture of these two types of growth (figure 11).

CONCLUSIONS.

1. The transplantable sarcomata of rats and mice grow very readily by the method of cultivating tissues *in vitro*.

2. Sarcomatous tissue grows in conformity to a type which may be regarded as characteristic for tissues of mesenchymal origin.

3. The growth of sarcoma cells *in vitro* consists in ameboid wandering into the surrounding plasma, karyokinetic proliferation, and evidences of active metabolism on the part of the cells.

4. Mouse carcinomata can be cultivated *in vitro*. The outgrowth of carcinoma cells assumes a sheet-like form, only one cell in thickness. They migrate into the plasma by ameboid movement, the advancing edge showing numerous prolongations of the cytoplasm into pseudopods.

5. Karyokinetic figures are frequently seen in growing carcinoma cells. The cells show evidences of active metabolism.

6. Both sarcoma and carcinoma cells cultivated *in vitro* show active phagocytosis; carmin particles placed in the plasma are taken up rapidly by the growing cells.

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EXPLANATION OF PLATES.

PLATE LXIII.

FIG. 1. Eighteen hour culture of rat sarcoma, stained with iron hematoxylin and Sudan III.

PLATE LXIV.

FIG. 2. Four day culture of rat sarcoma showing the ring form of growth, stained with iron hematoxylin and Sudan III. The ameboid wandering of the cells into the plasma is well shown.

PLATE LXV.

FIG. 3. Horizontal section of a thirty-six hour culture of a rat sarcoma stained with hematoxylin and eosin. Cells have wandered from the original piece of tissue into the coagulated plasma, the fibrin meshwork of which is seen.

PLATE LXVI.

FIG. 4. High power detail from the preparation illustrated in figure 1. The cells contain numerous granules of fat.

FIG. 5. Sarcoma cells growing *in vitro,* showing many pseudopods.

PLATE LXVII.

FIG. 6. × 1,000. Altmann's fixative and stain. Two mouse sarcoma cells are shown. The fat granules are stained black by osmic acid; the protoplasmic granules are colored with fuchsin. The fat granules are discrete and are of the same size as the protoplasmic granules.

PLATE LXVIII.

FIG. 7. × 1,000. Mouse sarcoma cells. The cells marked A and B contain carmin particles which they have taken up from powdered carmin added to the plasma. In cells C and D the protoplasmic granules have been colored by the carmin. All the cells exhibit vacuoles which in the living cell contained fat; the fat has been dissolved in the process of clearing the preparation.

PLATE LXIX.

FIG. 8. Three day culture of rat sarcoma showing the ring form of growth, stained with iron hematoxylin and Sudan III.

PLATE LXX.

FIG. 9. Five day culture of mouse carcinoma, stained with iron hematoxylin and Sudan III. The cells have migrated into the plasma in a sheet which is only one cell in thickness, except in the advancing edge, where there is a heaping up of cells. The hyaline ameboid fringe of cytoplasm is seen very clearly.

PLATE LXXI.

FIG. 10. × 1,000. A high power drawing of the edge of a four day culture of mouse carcinoma, stained with iron hematoxylin and Sudan III, showing the prolongation of the cytoplasm into pseudopods. One cell contains a karyokinetic figure. Numerous granules of fat are seen in the cytoplasm. The boundaries of the cells are very indistinct, in many places indiscernible.

PLATE LXXII.

FIG. 11. Several small groups of carcinoma cells, stained with iron hematoxylin and Sudan III, are shown, which have grown from a small detached piece of carcinomatous tissue. The growth of the carcinoma cells simulates the alveolar arrangement of carcinomatous growths in the body. The larger piece of implanted tissue is shown as a darkly stained area at one side. From this larger piece, many small, isolated cells have wandered into the plasma. These small ameboid wandering cells conform to the type of growth which we have described as characteristic of mesenchymal tissue. They are in all probability stroma cells.

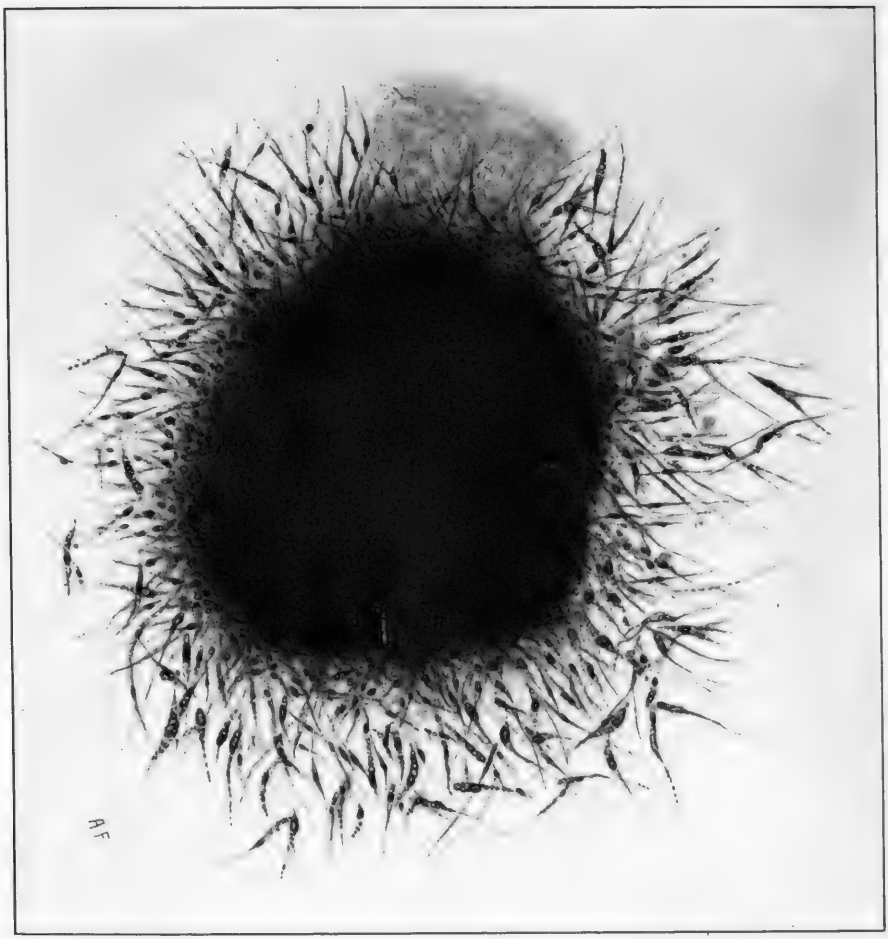

FIG. 1.

Mau

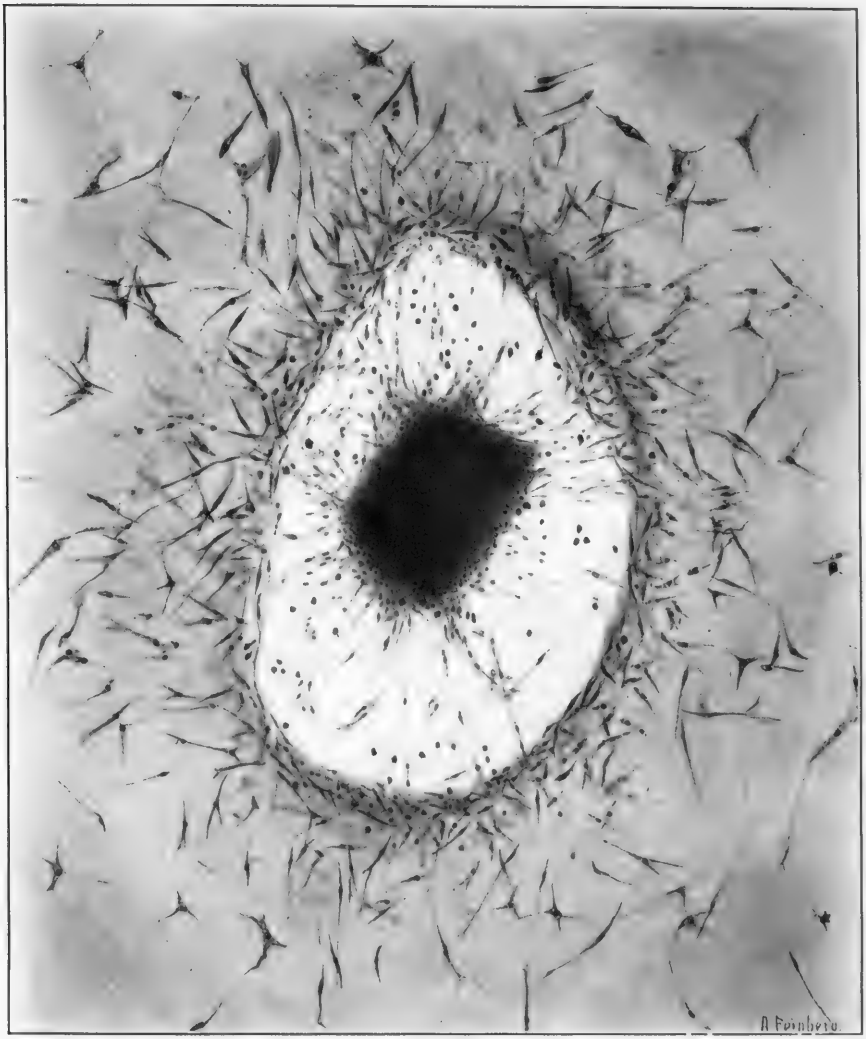

FIG. 2.

FIG. 3.

Mau

FIG. 4.

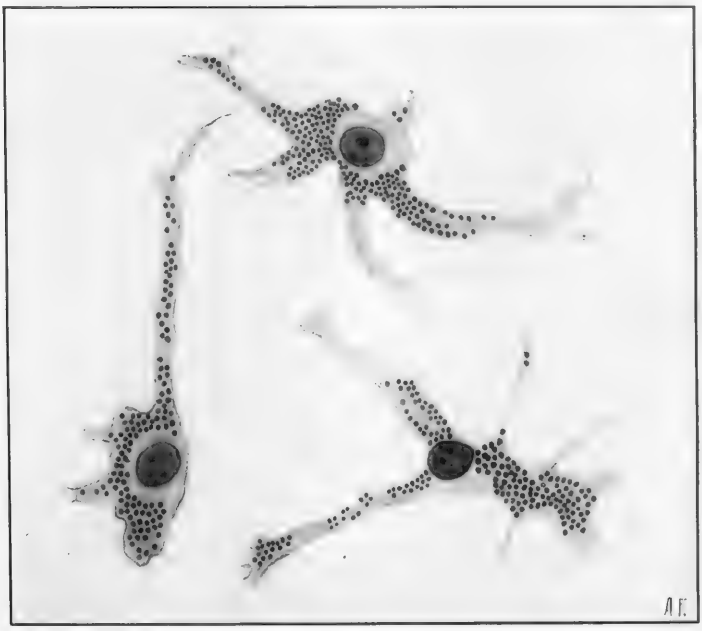

FIG. 5.

FIG. 6

FIG. 7.

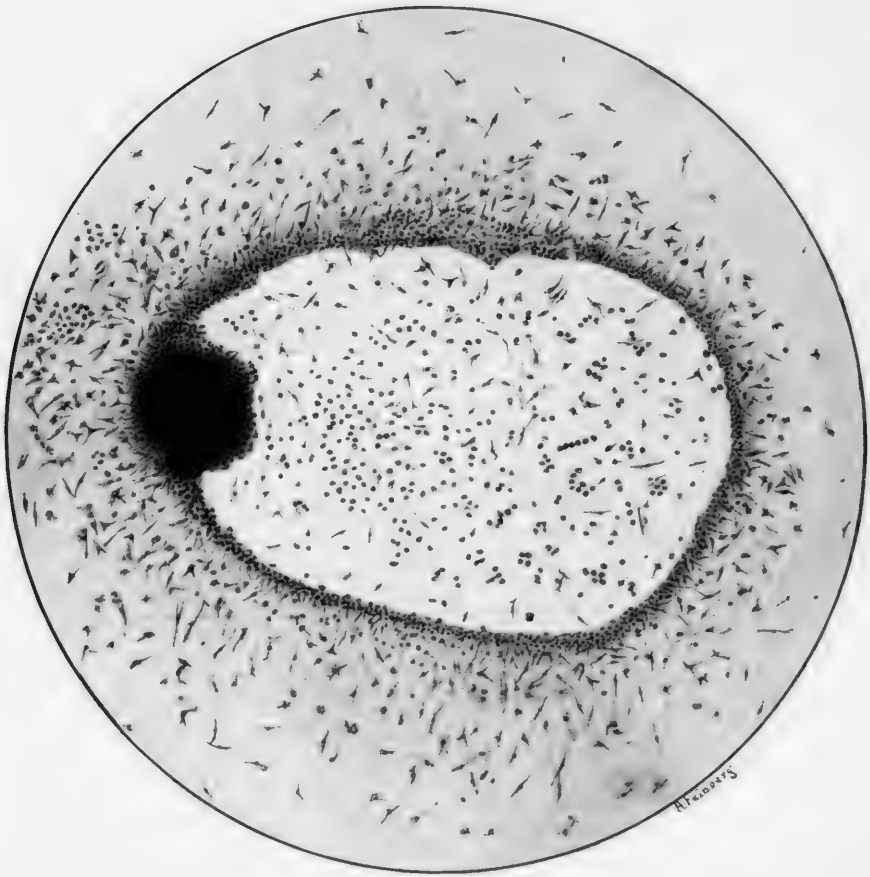

FIG. 8.

FIG. 9.

U of M

Mrau

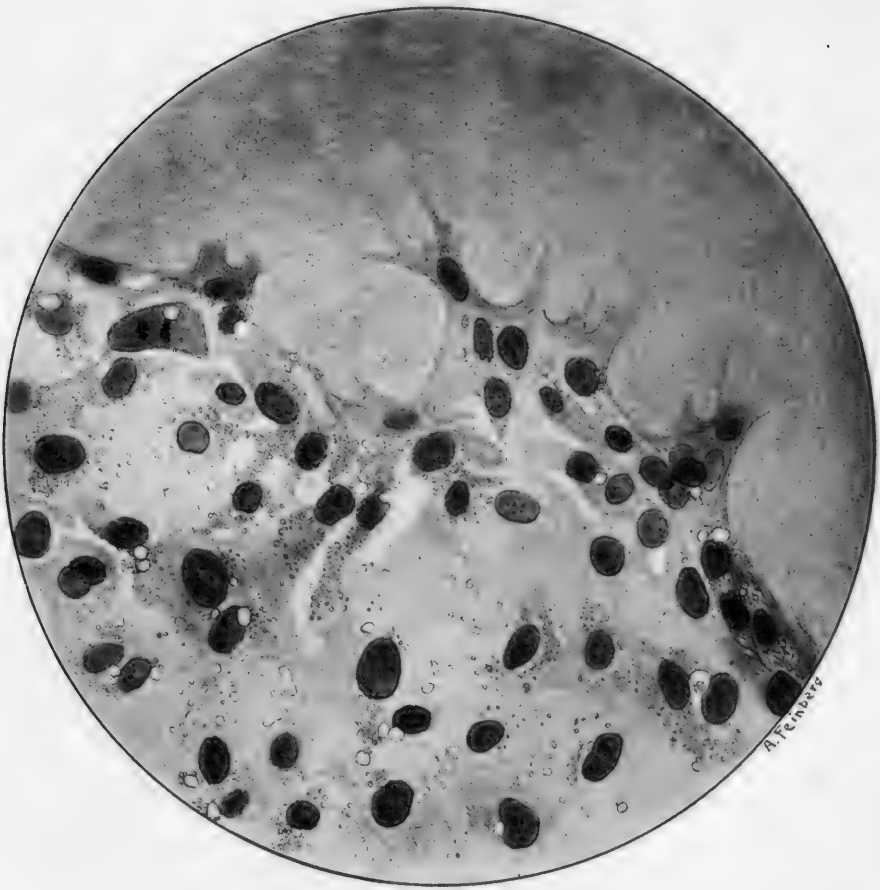

FIG. 10.

FIG. 11.

A STUDY OF CANCER IMMUNITY BY THE METHOD OF CULTIVATING TISSUES OUTSIDE THE BODY.*

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The phenomena of resistance and susceptibility to the transplantable tumors of rats and mice have been exhaustively studied. Extended observations have been made on the behavior of carcinoma grafts in immune mice. All of the recognized methods for the demonstration of specific antibodies have been employed in the study of the body fluids of immune animals. It may be stated summarily that immunity to tumor growth does not correspond to any of the types of immunity produced by the reaction of the body to injections of alien cells or to infective organisms and their products. Moreover, Russell (1) has shown that in immune mice inoculated cancer tissue fails to grow because of the absence of a specific stroma reaction on the part of the host, and not because of the presence of cytotoxic or cytolytic substances acting on the implanted grafts. Or, stated differently, in immune animals implanted cancer cells lose their power of calling forth a stroma reaction. He observed that, notwithstanding the absence of a stroma, the tumor cells remained alive and proliferated for eight to ten days.

The method of cultivating tissues outside the body, devised by Harrison (2) for the study of the developing nerve fiber in frog embryos, and modified by Burrows (3) in its application to warm-blooded animals, affords another means for studying the reaction of immunity to cancer, one, too, in which any reactive phenomena may be observed in all stages in a single specimen. Tumor cells growing in blood plasma find in the fibrin network an artificial stroma, so that the influence of the plasma alone may be held accountable if cancer cells which grow very readily in plasma from

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normal animals fail to grow or are modified in their growth in plasma from immune animals. In the present investigation we have applied this method to the study of tumor immunity, using a virulent rat sarcoma from Ehrlich's laboratory. It may be well, before describing our experiments, to review very briefly the subject of cancer immunity.

Two kinds of immunity to the transplantable rat and mouse tumors are recognizable, natural and artificial. The percentage of naturally immune animals (nullers) varies with age and with different varieties and strains. Old animals are, as a rule, less susceptible than young. Animals in which tumors have grown for a time and then undergone retrogression and absorption are highly resistant to further inoculations. This refractory condition may be regarded as a natural resistance artificially increased through the absorption of tumor tissue. Animals may be artificially immunized in various ways. Ehrlich (4) showed that mice inoculated with large doses of a hemorrhagic mouse carcinoma of very low virulence were rendered immune to more virulent carcinomata, and to a less extent to sarcoma. Loeb (5) was able to immunize rats in a similar manner, that is, by the inoculation of tumor tissue of artificially diminished resistance (heat). Others have shown that different degrees of resistance may be induced by the injection of suitable quantities of various homologous tissues, as, embryo (Schöne (7)), blood (Bashford (6)), liver (Bridré (8)), etc. Woglom (9) found that the subcutaneous inoculation of a mouse's own spleen gave results comparable to those obtained when spleen tissue from other mice was used. More recently, successful immunization through the use of tissues from alien species has been reported (C. Lewin (10), I. Levin (11)). Furthermore, I. Levin (11) has found that the autolyzed products of normal organs are quite as efficient as the fresh intact cells in the production of immunity. Ehrlich's athreptic immunity, to any wide application of which many objections have been advanced, constitutes another kind of immunity, but since it has no direct bearing on our experiments, a discussion of it will be omitted.

In the present study we have used six types of immune animals: (1) nullers from a strain of white rats in which sarcoma inocula-

tions gave 80 to 90 per cent. of takes; (2) nullers from a hooded variety of rats in which the percentage of takes was only 20 to 30 (racial immunity); (3) immune old animals whose immunity was probably attributable to their age; (4) animals in which both sarcoma and carcinoma inoculations had been negative (panimmunity); (5) animals whose tumors (sarcoma) had undergone retrogression and absorption; (6) artificially immunized rats from a series of animals that had received two cubic centimeters of rat's blood, and had resisted subsequent sarcoma inoculations. The percentage of resistant rats in the series was 40.

In all, eighteen immune rats were used, and from each sufficient plasma was obtained for fifteen to twenty culture preparations. We found that sarcoma grew readily in the plasma from every animal used. The character of the growth did not differ essentially from that observed in control specimens in which plasma from normal and tumor-bearing animals was employed.

ANIMAL INOCULATIONS.

To test the viability of sarcomatous tissue cultivated *in vitro*, animal inoculations were made. Chart 1 shows graphically the tumors resulting from the implantation of pieces of sarcoma that had been incubated for three days at 37° C., and were growing satisfactorily. In this series both the original pieces and the outgrowths embedded in plasma were used for inoculation. We have, however, obtained tumors when only the outgrowth was inoculated. Chart 1 is also designed to present a comparison of the tumors resulting from the inoculation of pieces of sarcoma cultivated in plasma from normal animals and of tumors from pieces grown in immune plasma. It is seen that the percentage of takes is as high, and the rate of growth as rapid, in the one as in the other. If, however, we compare the entire series of inoculations with sarcoma cultivated *in vitro* with a series of inoculations with sarcoma taken directly from the animal bearing the tumor, as is done in routine laboratory transplantations, we find two differences: first, the percentage of takes is 50 as compared with 80 to 90 in the inoculations with fresh tissue; and secondly, the number of late regressing tumors is about twice as great in the series from cultivated tissues.

The lowered percentage of successful inoculations is not, in this instance, necessarily an evidence of diminished virulence, since the pieces used were at least ten times smaller than those ordinarily

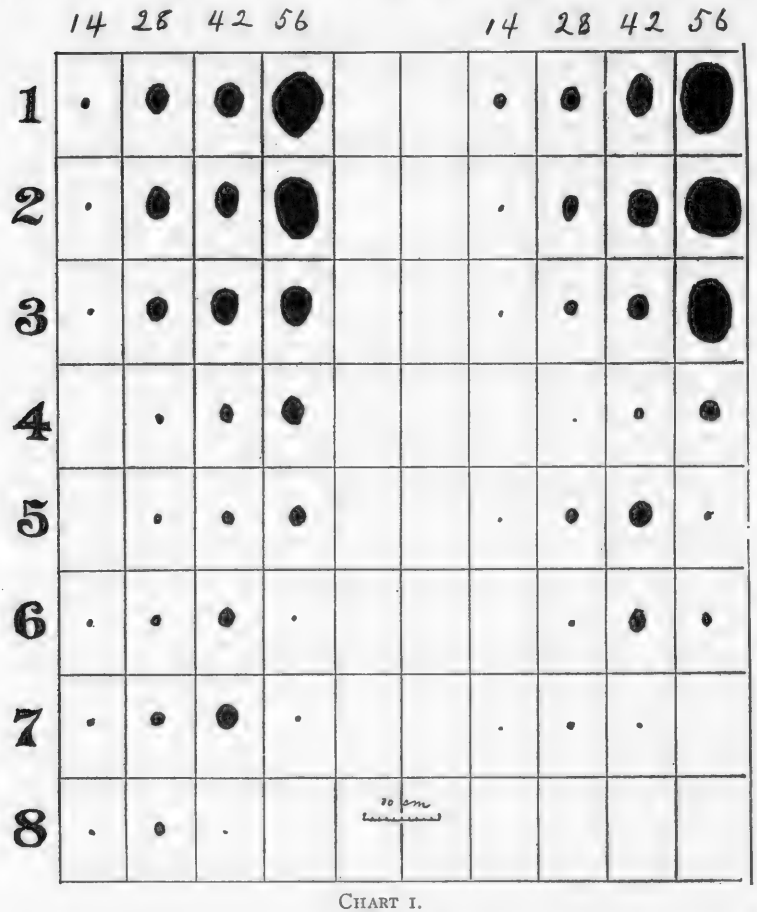

CHART I.

CHART I. The tumors on the left resulted from the inoculation of pieces of rat sarcoma cultivated three days in plasma from *immune* animals. The tumors on the right occurred in animals inoculated with sarcoma cultivated three days in plasma from *normal* and *tumor-bearing* rats. In each series there were fifteen rats; those not developing tumors were not charted. Six retrogressing tumors are noted, three in each group. The first row of figures indicates the number of days after inoculation.

implanted. Furthermore, the mass of plasma about the pieces of cultivated sarcoma was possibly an obstacle to early vascularization of the grafts. In order to test this possibility, animals were inoculated with bits of fresh sarcoma embedded in plasma. The yield of tumors was slightly greater (60 per cent.) than was obtained from the pieces of cultivated sarcoma. The relatively large number of regressing tumors, however, can hardly be interpreted other than as an evidence of lowered virulence.

Inoculations of mouse sarcoma cultivated for two days in rat plasma showed also a smaller percentage of takes than was observed in the inoculations with fresh tissue, but no regressing tumors have been noted.

Small pieces of sarcoma placed in hanging drops of blood serum and incubated at 37° C. were inoculated after twenty-four hours (twelve animals), thirty-six hours (eight animals), forty-eight hours (eight animals), and ninety-six hours (eight animals). Three tumors resulted from the twenty-four hour specimens, and two tumors from the thirty-six hour specimens. The inoculations from the forty-eight hour and ninety-six hour preparations were negative. Sarcoma cells incubated in blood serum, then, live at least thirty-six hours. A larger number of inoculations, however, would be necessary in order to determine definitely the duration of life of sarcoma cells under these conditions. We have obtained tumors from the inoculation of pieces of sarcoma after nine days incubation in plasma.

SUBCULTURES.

We have made subcultures of specimens of sarcoma growing in plasma by transferring to new medium the original piece of tissue or a portion of the outgrowth. Strictly speaking, transplantation of the original piece to new plasma does not constitute a subculture. The majority of the specimens cultivated in a single medium do not grow longer than five or six days, but, if before cell death takes place they are transferred to fresh plasma, active growth is renewed. We have in this way carried pieces through five drops of plasma, making the transfer at intervals of three to five days. In these subcultures, plasma from both normal and immune animals was used, and growth was as vigorous in the one as in the other.

CONCLUSIONS.

1. Sarcoma cultivated in plasma from immune animals grows quite as vigorously as in plasma from normal and tumor-bearing animals, thus affording further proof of the absence of specific cytolytic substances in the body fluids of animals immune to transplantable cancer.

2. Animals may be successfully inoculated with sarcoma cultivated *in vitro,* although, in the case of rat sarcoma, there is evidence of diminished virulence.

3. Subcultures of sarcoma cultivated *in vitro* may be made by transferring to fresh plasma the original piece of tissue, or a portion of the outgrowth. The duration of life of sarcoma cells under these conditions seems dependent only on a renewal of the medium.

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STUDIES ON IMMUNITY IN CANCERS OF THE WHITE RAT.

The Significance of Athrepsia.*

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In a previous communication, one of the present writers (1) showed that the result of tumor implantation is determined by two factors: first, whether or not the implanted tumor becomes ingrafted, and second, the rate of its growth if ingrafted.

The mechanism of ingrafting is common to tumor and normal tissue. Thus, Ribbert (2) has successfully implanted human skin into the ear of the rabbit. On the other hand, the ultimate fate and character of the graft depends on the rate of growth of the implanted tissue.

If the growth of the latter proceeds at approximately the same rate as that of the surrounding tissue, the implanted tissue develops into a small nodule which is ultimately absorbed. On the other hand, if the intensity of the cell proliferation in the implanted tumor exceeds that of the surrounding tissue, there will develop a large malignant tumor. The rate of growth of an implanted tumor and with it the ultimate character of the tumor depend upon an interaction between the power of proliferation of the tumor cell and the resistance of the host. The mere grafting of the tumor is independent of the phenomenon of cancer immunity.

A great deal of the confusion and controversy that exists in the attempted analysis of the results of the work on cancer immunity is caused by this lack of appreciation of the coexistence of the two factors determining the outcome of the tumor implantation. It seemed, therefore, desirable to undertake a revision of the work on cancer immunity.

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The most popular and most generally accepted theory of cancer immunity is the one advanced by Ehrlich and is commonly designated the athrepsia hypothesis. It is based on the following considerations:—

Immunity or resistance of the host to the growth of an inoculable cancer is not specific in its character, and an animal immune against carcinoma will be also immune against sarcoma. The methods used to induce an artificial immunization are likewise not specific. When a cancer of low virulence is inoculated into and absorbed by the host, the latter is immune in the majority of cases against subsequent inoculation of any of the most virulent cancers. The same immunization may also be accomplished by an inoculation of normal tissue cells of the same species of animals or even of cells of a phylogenetically closely related species of animals, as, for instance, with mouse cells in immunization of the rat, and *vice versa*. This non-specific character of cancer immunity Ehrlich designates by the name of panimmunity.

According to Ehrlich, as well as to most of the investigators on the subject, an artificial immunity may be induced only by treatment with living cells. Growth of inoculated cancers is due, of course, to cell activity. Ehrlich contends that the growth of a cancer indicates that the tumor cells possess a great avidity for a certain specific food within the organism of the host and consequently obtain it from the normal tissue cells of the organism. When cancer fails to grow, then either the organism of the host does not possess the necessary specific food or else the avidity of the cancer for this food is not strong enough to deprive the normal tissue cells of it. When the organism of the host is immunized by treatment with a cell emulsion, these cells bind the specific food and consequently the cancer cells inoculated subsequently do not find the necessary nourishment and die. Immunity, then, to cancer growth consists in the lack of food athrepsia.

The experimental evidence adduced by Ehrlich in support of his theory consists in the so-called zigzag transplantation, the second inoculation on a tumor-bearing animal and the inoculation of an emulsion consisting of a mixture of two tumors (carcinoma and sarcoma).

In the present investigation, a separate detailed study was made of each of the experimental proofs mentioned above, and in the following pages each of them will be considered separately. The studies on immunity in inoculable cancer, of which this investigation presents one phase, were conducted on white rats only, as these animals are better adapted for experimentation. For the present investigation, however, both rats and mice were used, and in all five different tumors served for the experiment. Two of these were rat tumors; an extremely virulent Ehrlich sarcoma, and the Flexner-Jobling carcinoma, which is not as virulent as the former. The three mouse tumors used were Ehrlich's carcinoma of the mouse (No. 33), Bashford's carcinoma of the mouse (No. 27), and Ehrlich's sarcoma of the mouse. The last named was the most virulent of the mouse tumors.

PANIMMUNITY.

Ehrlich did not consider the phenomenon of panimmunity a direct proof of athrepsia, but there must undoubtedly be a certain relation between the general non-specific character of the former and athrepsia, which is merely a phase in intracellular nutrition. Indeed, if athrepsia is to have a general application it must be capable of explaining the conditions of panimmunity against the different tumors used in this research, and it may be stated that in a general way the fact of panimmunity was found to be correct for the majority of cases. But there were factors observed in the course of these experiments which can not be explained on the basis of the theory of athrepsia. Of such experiments the following instances may be cited.

Experiment 1.—Twenty-nine rats which had been previously immunized against an inoculation of a rat carcinoma (Flexner-Jobling) were inoculated four weeks later with a rat sarcoma (Ehrlich). Of these rats, twenty-three remained immune and only six, or 20 per cent., of the inoculated animals grew the tumor, while of twenty control rats, 17, or 85 per cent., took the tumor.

Of these two tumors, the Flexner-Jobling carcinoma grows much more slowly than the Ehrlich rat sarcoma and does not reach as large a size. Consequently the former tumor has less avidity for the specific food than the sarcoma, and does not, according to the Ehrlich theory, use up all this food of the host. Therefore, a suffi-

cient amount of specific food should be left for the subsequently inoculated sarcoma cells, and it should be possible to inoculate the latter successfully in all the treated animals.

The following experiment shows again that all the facts of panimmunity can not be explained on the basis of athrepsia.

Experiment 2.—Twenty rats were inoculated with a mouse sarcoma, and four weeks later, when the grafts were completely absorbed, they were inoculated with a rat sarcoma. Of the fifteen rats which survived at the last examination, the tumor grew only in two. This shows 13 per cent. of takes, while in the twenty control animals, the tumor took in 90 per cent.

It will be shown later that according to the theory of athrepsia the cells of a mouse tumor are unable to anchor the specific food of the rat. Consequently the specific food needed for the success of the inoculation of a rat sarcoma should exist in the same quantity in the rats treated with mouse sarcoma as in the control animals. Still the rats treated with mouse tissue were immune to the inoculation of rat tumor. The fact that it is possible to immunize a rat with mouse tissue, and *vice versa*, was previously observed by Carl Lewin and by one of the present writers.

Another factor observed in the course of these experiments on panimmunity, which is of great importance in the consideration of the significance of athrepsia, is the following: whenever a tumor succeeds in growing in one of the previously treated animals, it attains the same size and virulence as in the control animals.

This phenomenon indicates that panimmunity with its non-specific character is in reality an immunity to tissue grafting. On the other hand, when the cancer cells are ingrafted, such preliminary treatment of the animals with living tissue, as is used in experiments on panimmunity, does not exert any influence whatever on the subsequent growth or virulence of the developing tumor. The truth of this assertion is also evident from the fact that no immunization with living tissue is successful if it is done subsequently to the inoculation of the tumor.

The experiments of Rous (3) with inoculation of embryonic tissue show that by the same method an animal may be immunized against the grafting of embryonic cells. Here again is apparent the non-specificity of immunity in cancer. The panimmunity is in

reality an immunity against implantation of tissue. But since in all experimental cancer work with the inoculable tumors the first step always consists in the inoculation of a piece of the tumor, such tissue immunity obscures the actual immunity to the growth and development of the cancer.

ZIGZAG TRANSPLANTATIONS.

This phenomenon, which served as the main experimental basis for the formation of the theory of athrepsia, consists in the following: When a mouse sarcoma is inoculated into a rat, it grows there normally for about eight or ten days, then ceases to grow and is absorbed. But if before absorption takes place the nodule is removed from the rat and transferred to a mouse, it continues to grow normally and may be subsequently transferred to another rat, where it will grow for about eight days, and so on.

Ehrlich explains the result of these experiments by the supposition that tumor cells in order to proliferate must obtain a certain specific food x, which they can find only in an animal of the same species as that from which the tumor cells have come. Cells of a mouse tumor inoculated into a rat proliferate for a few days, as long as the food x, which they brought over from the mouse, lasts. When this is used up, they do not find this specific food in the rat and cease to grow, but grow again when transferred to a mouse, where they find again this specific food. On the basis of this supposition, Ehrlich created the general theory that whenever cancer cells fail to proliferate, it means that they fail to obtain the food x, either because the normal body cells have greater avidity for this food than the cancer cells, or else the cells with which the animals were immunized anchored all the specific food and the cancer cells inoculated subsequently could not obtain it.

The experiments of zigzag transplantation were undertaken by the writers on all the tumors used in this investigation. In the course of these experiments certain facts were observed which indicate that athrepsia can not account for all the phenomena observed with zigzag transplantations.

The inoculation of the rat sarcoma and rat carcinoma into mice and of the Ehrlich mouse carcinoma into rats uniformly produced

small nodules in the host of different species. When these nodules were retransferred into animals of the species from which the original tumors were derived, for instance, when a rat sarcoma was retransplanted from a mouse into a rat, not a single good tumor growth could be produced by any of the three tumors mentioned above.

With Ehrlich's mouse sarcoma and Bashford's mouse carcinoma, the results of the experiments appeared to be more successful, i. e., when a nodule of the mouse sarcoma or carcinoma, after a sojourn of eight to ten days in a rat, was removed and inoculated into a mouse, it grew, in a certain number of cases, into a good sized tumor. The number of such successful inoculations, however, was much smaller than the number of takes in control animals, i. e., in animals that were inoculated with tumor material which was taken directly from a mouse. The following series of experiments illustrates this point.

Bashford's Mouse Carcinoma Inoculated into 20 Rats; 2, 4, 6, and 8 Days Later Nodules Were Removed from the Rats and Inoculated into Mice.

	2 days later.	4 days later	6 days later.	8 days later.	Total.	Control.
No. of mice inoculated	10	10	15	10	45	20
No. of mice surviving at final examination	8	9	14	9	40	20
No. of mice with tumors	4	1	4	1	10	18
Per cent. of takes					25	90

Ehrlich's Mouse Carcinoma Inoculated into 20 Rats; 2, 4, 6, and 8 Days Later Nodules Were Removed from the Rats and Inoculated into Mice.

	2 days later.	4 days later.	6 days later.	8 days later.	Total.	Control.
No. of mice inoculated	10	10	10	7	37	20
No. of mice surviving at final examination	9	6	10	1	26	18
No. of mice with tumors	1	2	0	1	4	14
Per cent. of takes					15.4	77.8

An analysis of these experiments on zigzag transplantations shows that all tumors, or at least all the tumors with which this investigation was conducted, can be successfully grafted on the phylogenetically closely related animals, i. e., from rat to mouse and *vice versa*. The same holds true, according to Rous, for em-

bryonic tissue. But the ingrafted cancer cells do not proliferate indefinitely and become malignant in the host of a foreign species. The explanation of this phenomenon given by Ehrlich does not seem to be adequate to explain all the factors in connection with it. If a mouse tumor fails to grow in the rat only for lack of specific food, then it should retain its normal virulence when transferred into a host of its own species. But experiments show that the virulence of a mouse or rat tumor is so diminished by its eight to ten days sojourn in a host of a different species that it either fails to take at all when reinoculated into a host of its own species, or it takes in a very small percentage. It is evident then that the organism of the temporary host has a decidedly noxious effect upon the proliferating activities of the tumor cells, and, moreover, while the capacity for ingrafting in an animal of a foreign species is common to all the tumors studied and is non-specific, the amount of injury sustained through the sojourn in the foreign host is different with the different tumors.

SECOND INOCULATION ON A TUMOR-BEARING ANIMAL.

Ehrlich maintains that animals on which a successful inoculation of another tumor was previously made appeared to be immune against a subsequent inoculation, either of the same or another tumor. The opposite results found by Hertwig and Poll (4), Gierke (5), and Borrel (6), he explains by the fact that in their experiments they used for the primary inoculation tumors of low virulence and slow growth, while in his experiments the animals received for the first inoculation an extremely virulent tumor. Only a very virulent, rapidly growing tumor anchors all the specific food x and consequently makes the successful take of the subsequently inoculated tumor impossible. A slowly growing tumor leaves a sufficient amount of the specific food for the successful take of a subsequent inoculation.

Experiments with a double inoculation were conducted with the Flexner-Jobling rat carcinoma and with Ehrlich's rat sarcoma. The latter is an extremely virulent and rapidly growing tumor, and still a subsequent inoculation of the carcinoma was just as successful on an animal bearing a large sarcoma as the inoculation of

the sarcoma on an animal with a growth of carcinoma. The most plausible explanation of the discrepancies in similar experiments of different investigators consists in the different behavior of the different tumor cells after a successful grafting. This latter factor is most evident in the series of experiments on the inoculation of an emulsion consisting of two tumors.

INOCULATION OF AN EMULSION CONSISTING OF TWO TUMORS.

Apolant (7) inoculated an emulsion of a carcinoma and sarcoma into an animal and developed a mixed tumor showing the structure both of the carcinoma and sarcoma. If one of the tumors was originally more virulent than the other, or if its virulence was increased artificially, then the resulting growth showed the structure of the more virulent tumor only. Ehrlich adduces the results of this experiment as another proof of the fact that the more virulent tumor cells obtain all the specific food of the host, causing the cells of the weaker tumor to die of starvation.

These experiments were repeated with two emulsions. One, consisting of the cells of the Flexner-Jobling carcinoma and of Ehrlich's sarcoma of the rat, was injected into rats, and another, consisting of Bashford's carcinoma and Ehrlich's mouse sarcoma, was injected into mice. In both instances the sarcoma was a great deal more virulent than the carcinoma. The relation in virulence between the Flexner-Jobling carcinoma and the Ehrlich rat sarcoma, on the one hand, and the Bashford carcinoma and the Ehrlich mouse sarcoma, on the other, is quite identical, but a most surprising difference was observed in the results of the two experiments. While in the rats, on the injection of the emulsion, there developed a mixed tumor showing the structure both of carcinoma and sarcoma, the tumor in the mice appeared to be pure sarcoma. Thus it seems that different tumors behave in a different manner when introduced simultaneously into an organism.

Of all the experiments stated above, the zigzag transplantations undoubtedly offer the best field for the analysis of the different phenomena of immunity in cancer. The apparent success of an inoculation of a mouse tumor into a rat may mean only the success of the initial grafting, since the rat possesses a congenital racial im-

munity against the growth of a mouse cancer. Indeed, the nodule which develops in the rat upon an inoculation of a mouse tumor never develops into a large malignant growth. There is consequently a condition here in which the dual character of the immunity to an inoculable tumor is perfectly apparent. The rat is susceptible to the grafting of a mouse sarcoma, but immune against its malignant development. Another proof of these two conditions in cancer immunity is shown in the influence of pregnancy on inoculations of a tumor. Haaland (8) found that an inoculable tumor does not take well on a gravid animal, and Ehrlich explains this fact on the supposition that during pregnancy all the available food is used up by the body cells. Herzog (9) has shown, on the other hand, that when a tumor-bearing animal becomes pregnant the tumor appears to grow faster. In other words, pregnancy produces a resistance against the grafting of tumor cells, while it enhances the proliferation of the tumor cells present in the organism.

The success of the grafting of the inoculated cells undoubtedly depends upon conditions of nutrition. Thus, athrepsia may serve to explain the conditions of resistance and susceptibility to the grafting of tumor as well as tissue cells. The formation of a highly vascularized stroma around the successfully ingrafted tissue and the lack of stroma formation in an unsuccessful graft (Russell (10)) also shows the importance of nutrition during this phase of the inoculation of a tumor.

On the other hand, the same experiments on zigzag transplantations clearly indicate that differences in nutrition can not explain the reason why in one successful tumor graft a small nodule will form which is subsequently absorbed, while in another the nodule will grow into a large tumor and kill the animal.

As stated above, this investigation has shown that a lack of specific food alone can not explain the failure of a mouse carcinoma or sarcoma to develop into a malignant tumor in the rat. The organism of the rat has a direct influence upon the mouse tumor and its virulence is greatly diminished after a sojourn of a few days in this animal. The organism of the rat has consequently a noxious effect upon the proliferating activities of the cells of the mouse tumor. It is of great interest to note in this connection that

upon an inoculation of these weakened cells into another generation of mice, the normal virulence of the former returns. The following series of experiments illustrates this point.

Experiment 3.—Twenty mice were inoculated with pieces of the Ehrlich mouse sarcoma taken from rats. Nineteen mice survived at the last examination, and of these, one took the tumor, which represents 5 per cent. of takes. The tumor from this mouse was inoculated in twenty other mice and the tumor took in fourteen, or 70 per cent.

It appears from these experiments that a mouse tumor through a sojourn in the rat reacquires the characteristics of a spontaneous tumor; namely, it takes in a small percentage of inoculated animals, and this percentage increases in subsequent generations. But the diminution of the virulence of the tumor which takes place in the organism of the rat is due to congenital racial immunity of the latter. Furthermore, the immunity of the rat against mouse tumor is not due to conditions of nutrition, but is accompanied by an active noxious influence of the organism of the rat on the mouse tumor. Thus indirect evidence is brought forward through this investigation to show that immunity to cancer growth, but not to tumor grafting, is caused not by differences in intracellular nutrition, but by an active inhibitory influence of the organism of the host on the cancer cells. The search for direct evidence and the true nature of this inhibitory influence is the aim of further studies on immunity in cancer of the white rat.

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THE RELATION OF CERTAIN BACTERIA TO NON-SPECIFIC REACTIONS WITH THE COMPLEMENT FIXATION TEST FOR LUES.*

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Several investigators who have had a large experience with the complement fixation test for lues have called attention to the development in sera of anticomplementary bodies which cause inhibition of hemolysis in both the antigen and control tubes, and they have found that in most instances this property of the serum is lost after heating it for half an hour at 56° C. In some instances, however, the property still persists after heating, and in such sera one is unable to read the reaction. It would appear from these observations that the anticomplementary bodies are of two kinds, thermolabile and thermostabile, and that the inactivation of serum enables us to destroy only the thermolabile group.

The exact nature of these anticomplementary substances is still in doubt, but it is the purpose of this contribution to show that certain strains of bacteria, if growing in human blood serum, are capable of producing total inhibition of hemolysis in both antigen and control tubes; and that not only is this true, but certain bacteria also possess the power of producing inhibition in the antigen tubes alone, thus giving rise to a non-specific reaction in the sera which contain them.

In the application of the complement fixation to the diagnosis of lues in the military service, we have adopted in this laboratory the modification of the Wassermann test devised by Noguchi, the only departure from his technique being the use of an alcoholic extract of fetal luetic liver as antigen, and the inactivation of the serum

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before testing. As we make tests for many army posts at some distance from this laboratory, the use of active serum is impossible, as most of the serum reaching us is at least two, and often from three to four days old. For this reason, and in order to avoid the thermolabile anticomplementary bodies and the non-specific reactions sometimes observed when an alcoholic extract is used as antigen, the sera are always inactivated just before making the test. The results we have obtained in over 2,000 complement fixation tests have justified the technique employed, and have shown that, with the Noguchi modification of the Wassermann test, it is possible to use sera several days old with as good results as fresh sera, provided that bacterial contamination is avoided, and that the sera are inactivated before the test is made. Serum in which hemolysis has occurred can not be used on account of the difficulty of reading the reaction; so it is important that the serum be allowed to separate thoroughly from the clot before it is sent to the laboratory.

Before adopting the Noguchi modification of the complement fixation test in the army, we conducted careful experiments upon the keeping properties of human sera, especially as some authorities had reported that certain specimens of normal serum, if kept for several days, would give a positive reaction. Our experiments convince us that as long as normal sera remain free from bacteria they do not give a positive reaction, even though kept at room temperature for a month, although in some of the sera experimented with, both thermolabile and thermostabile anticomplementary bodies developed, which caused inhibition of hemolysis in both antigen and control tubes.

In all, fifty normal sera were tested, the blood being collected in Wright tubes and the serum allowed to separate, after which it was pipetted into suitable glass containers and sealed. Enough of these tubes were prepared to enable us to make a complement fixation test upon each serum at intervals of one week for a month. Of the fifty sera examined, all gave a negative reaction at the time of collection and forty-three were still negative at the end of a month, while seven had developed anticomplementary bodies which produced complete inhibition of hemolysis in both antigen and control tubes. As an alcoholic extract was used as antigen, the sera were

always inactivated before the test was made, but in two sera tested before inactivation, both being about two days old, a positive reaction was obtained. Both of these sera gave a negative reaction after inactivation, and neither was contaminated by bacteria. This observation confirms that of other investigators who have found that non-specific reactions may occur in testing active sera when an alcoholic extract is used as antigen.

Our results in this series of experiments convince us that a positive reaction does not occur in normal sera for a period of time far in excess of any delay that may occur before the specimens reach the laboratory, provided that proper care is taken in collecting them, and that the sera are inactivated before use. The following table (table I) gives the results obtained in one series of experiments and is illustrative of all with normal sera free from bacterial contamination.

TABLE I.

*Complement Fixation in Normal Sera, Kept at Room Temperature for One Month.**

No. of serum.	Date.	Result.	Date.	Result.	Date.	Result.	Date.	Result.
1	Jan. 13	—	Jan. 21	—	Jan. 28	—	Feb. 4	—
2	"	—	"	—	"	—	"	—
3	"	—	"	—	"	—	"	—
4	"	—	"	—	"	—	"	—
5	"	—	"	—	"	—	"	—
6	"	—	"	—	"	—	"	—
7	"	—	"	—	"	—	"	—
8	"	—	"	—	"	—	"	—

* The blood was collected in Wright tubes. The serum was allowed to separate and was then transferred to glass tubes and sealed, one tube being opened for each test.

The importance of the prevention of bacterial contamination of the sera was recently emphasized in the case of a specimen sent from San Francisco to this laboratory during some experiments we were making upon the effect of transportation on serum submitted for the complement fixation test. Arrangements were made with the laboratory of the U. S. Army General Hospital at San Francisco, California, to send us each week duplicates of sera that were tested there, in order that we might repeat the tests and thus ascertain whether any changes had occurred in the sera during trans-

portation. All the sera sent us from San Francisco were at least six days old when received, and the following table (table II) gives the results obtained in both laboratories.

TABLE II.

Results of Comparative Complement Fixation Tests upon 80 Sera at San Francisco and Washington, D.C.

Case no.	Test 1.		Case no.	Test 1.		Test 2.		Test 3.		Test 4.	
	S. F.*	Wash.*		S. F.	Wash.	S. F.	Wash.	S. F.	Wash.	S. F.	Wash.
1	−	−	41	?	?						
2	−	−	42	−	−						
3	+	+	43	?	?						
4	+	+	44	+	+						
5	−	−	45	+	+						
6	−	−	46	−	−						
7	+	+	47	−	−						
8	−	−	48	+	+						
9	−	−	49	−	−						
10	+	+	50	−	−						
11	+	+	51	+	+						
12	+	+	52	+	+	+	+	+	+	+	+
13	+	+	53	−	−	−	−	−	−		
14	−	+	54	+	+	+	+	+	+		
15	−	−	55	+	+	+	+	+	+		
16	+	+	56	−	−	−	−				
17	−	−	57	+	+	+	+				
18	−	−	58	+	+	+	+				
19	−	−	59	−	−	−	−				
20	−	−	60	+	+	+	+				
21	−	−	61	+	+	+	+				
22	−	−	62	−	−	−	−				
23	+	+	63	−	−	−	−				
24	+	+	64	+	+	+	+				
25	−	−	65	+	+	+	+				
26	−	−	66	−	−	−	−				
27	−	−	67	+	+	+	+				
28	−	−	68	+	o						
29	−	−	69	+	o						
30	+	+	70	+	o						
31	−	−	71	+	o						
32	−	−	72	+	o						
33	−	−	73	−	o						
34	+	+	74	+	o						
35	+	+	75	+	o						
36	−	−	76	+	o						
37	−	−	77	+	o						
38	+	+	78	−	o						
39	+	+	79	+	o						
40	+	+	80	+	o						

* S. F. = San Francisco. Wash. = Washington, D. C.

A consideration of the table shows that in sixty-seven of the eighty sera recorded the results were identical except in one in-

stance, a serum which gave a negative result at San Francisco and a positive one in this laboratory. There were thirteen sera in which no result could be obtained in this laboratory because of the development of thermostabile anticomplementary bodies during the interval of time elapsing between the test in San Francisco and that in Washington. In all of these sera, inhibition of hemolysis occurred in both antigen and control tubes, and all were contaminated with bacteria.

The occurrence of a positive result in one specimen of serum which was negative at San Francisco suggested that perhaps certain bacterial substances might have developed in the serum which gave a positive reaction, especially as it appeared cloudy and had a putrefactive odor. In order to determine this point it was plated out upon agar-agar and pure cultures of the following organisms were finally obtained: *Staphylococcus aureus;* a large *Staphylococcus albus;* a small *Staphylococcus albus;* a large spore-bearing bacillus of the subtilis type; and a very minute diplobacillus.

The following complement fixation experiments were undertaken with these organisms, and also with a stock culture of *Staphylococcus aureus, Staphylococcus citreus,* and *Streptococcus pyogenes.* The same technique was employed in these experiments as in the routine complement fixation tests made in the laboratory, the mixtures of serum and bacteria being substituted for pure serum.

TABLE III.

Result of Complement Fixation Tests in Experiment I.

Name of bacterium.	Result.		Controls.		Origin of Bacterium.
	Antigen tube.	Control tube.	Bacteria alone.	Serum alone.	
S. albus (large)	–	–	–	–	Isolated from serum.
S. albus (small)	–	–	–	–	" " "
S. aureus.....	–	–	–	–	" " "
Bacillus (spore).....	–	–	–	–	" " "
Diplobacillus.....	–	–	–	–	" " "
Combined bacteria. ...	–	–	–	–	" " "

Experiment I.—An emulsion in normal salt solution was made from a pure culture of each organism isolated from the serum, and four drops of the emulsion were placed in 1 c.c. of a 1 per cent. suspension of human red blood corpuscles, together with four drops of an inactivated normal serum. An emulsion was also made of all the bacteria combined, and four drops of this and four drops of

normal serum were added to the same amount of blood suspension. Table III gives the results obtained in this experiment.

From the results obtained in this experiment it is evident that merely adding an emulsion of the cultures of the bacteria to normal serum is without effect upon the result of the complement fixation test, as all of the mixtures gave a negative reaction.

Experiment 2.—This was identical with experiment I except that the mixtures of serum and bacteria were inactivated at 56° C. for one half hour before testing. The results are given in table IV.

TABLE IV.

Result of Complement Fixation Tests in Experiment 2.

Name of bacterium.	Result.		Controls.		Origin of bacterium.
	Antigen tube.	Control tube.	Bacteria alone.	Serum alone.	
S. albus (large)	−	−	−	−	Isolated from serum.
S. albus (small)	−	−	−	−	" " "
S. aureus	−	−	−	−	" " "
Bacillus (spore)	−	−	−	−	" " "
Diplobacillus	+	+	+	−	" " "
Combined bacteria	−	−	−	−	" " "

It would appear from this experiment that the heating of the mixture of serum and the diplobacillus resulted in the liberation of inhibitory substances sufficient to cause inhibition of hemolysis in both the antigen and control tubes. All the other mixtures gave a negative result.

Experiment 3.—Specimens of normal serum were inoculated with each of the bacteria isolated from the serum as well as with a stock culture of *S. aureus, S. citreus,* and *Streptococcus pyogenes,* and allowed to stand at room temperature for one week before testing. Table V gives the result of this experiment.

TABLE V.

Result of Complement Fixation Tests in Experiment 3.

Name of bacterium.	Result.		Control.	Origin of bacterium.
	Antigen tube.	Control tube.	Serum alone.	
S. albus (large)	+−	−	−	Isolated from serum.
S. albus (small)	−	−	−	" " "
S. aureus	−	−	−	" " "
Bacillus (spore)	−	−	−	" " "
Diplobacillus	+	++	−	" " "
S. aureus	++	−	−	Stock culture.
S. citreus	++	++	−	" "
S. pyogenes	−	−	−	" "

It is evident from this experiment that after incubation in human serum for one week at room temperature the large *S. albus* isolated from the serum under discussion gave a plus-minus reaction in the antigen tube, while the diplobacillus isolated from the same serum gave a plus reaction in the antigen tube, and a double plus reaction in the control tube. The stock culture of *S. aureus* gave a double plus positive reaction, while the stock culture of *S. citreus* caused total inhibition in both antigen and control tubes.

Experiment 4.—This was identical with experiment 3, except that the mixtures of serum and bacteria were incubated for twenty-four hours at 37° C. and then allowed to stand at room temperature for one week. Table VI gives the results of this experiment.

TABLE VI.

Result of Complement Fixation Tests in Experiment 4.

Name of bacterium.	Result.		Control.	Origin of bacterium.
	Antigen tube.	Control tube.	Serum alone.	
S. albus (large)	++	−	−	Isolated from serum.
S. albus (small)	−	−	−	" " "
S. aureus	++	−	−	" " "
Bacillus (spore)	−	−	−	" " "
Diplobacillus	++	++	−	" " "
S. aureus	++	−	−	Stock culture.
S. citreus	++	++	−	" "
S. pyogenes	++	−	−	" "

The results were surprising in that no less than four of the organisms tested gave a double plus positive reaction in the normal serum alone, while two inhibited hemolysis in both antigen and control tubes. It would appear that the twenty-four hours at incubator temperature lead to an increase in the intensity of the reaction, probably by favoring a richer development of the bacteria in the sera.

Considering the experiments as a whole, it is perfectly evident that a positive complement fixation reaction may be obtained in a normal serum if certain species of bacteria are enabled to develop within it. That every strain of a certain bacterial species will not produce this result is shown by the fact that while a double plus reaction occurred in the serum infected with the stock *S. aureus* and with the *S. aureus* isolated from the serum under discussion, a negative result was obtained with another *S. aureus* isolated from a serum which gave a negative reaction.

It is also evident that temperature has much to do with the ability of the bacteria to produce a non-specific reaction, for in the mixtures of serum and bacteria kept for twenty-four hours at incubator temperature, the reactions were most marked, while two of the organisms which did not give a reaction when growing in the serum at room temperature, gave strong positive reactions when incubated at 37° C. for twenty-four hours. Inactivation also appears to favor the production of a false positive reaction in sera which are contaminated with certain bacteria. As regards the San Francisco serum under discussion, it is evident that the positive result at this laboratory could have been caused by either the large *S. albus* or the *S. aureus,* or by both, according as the conditions at the time of the examination were favorable to the production of inhibitory substances by one or both of these organisms. The following is a summary of the action of each of the bacteria under the conditions of the various experiments.

The large *S. albus* isolated from the serum under discussion gave a negative result in both antigen and control tubes when added directly to normal serum, whether the mixture was or was not inactivated; when it was added to normal serum and kept at room temperature for a week and inactivated, it gave a plus-minus positive reaction; and finally, when added to normal serum, kept at incubator temperature for twenty-four hours and at room temperature for one week, the mixture being then inactivated, a double plus positive reaction was obtained. This organism was the most consistent in its behavior toward the complement fixation test of any of those examined, and its activity in giving a positive reaction seems to depend entirely upon conditions favorable to its growth in the serum.

The small *S. albus* from the same serum gave a negative result in both tubes under every experimental condition.

The *S. aureus* which was isolated from the serum, gave a negative result in both tubes under all conditions except those of experiment 4, in which it gave a double plus positive reaction. It is undoubtedly true that the more favorable conditions for growth in this experiment account for the result obtained.

The large spore-bearing bacillus gave a negative result in both tubes throughout the experiments.

The small diplobacillus inhibited hemolysis in both tubes except in experiment 1, where it gave a negative result in both tubes.

The stock *S. aureus,* under the conditions of experiments 3 and 4, the only ones in which it was tried, gave a double plus positive reaction.

The stock *S. citreus,* under the conditions of experiments 3 and 4, the only ones in which it was tried, gave complete inhibition of hemolysis in both tubes.

The stock *Streptococcus pyogenes,* under the conditions of experiment 4, gave a double plus positive result, but a negative result under the conditions of experiment 3. Here again the result, as regards a positive reaction, appeared to depend upon favorable cultural conditions.

Under the conditions of experiment 4, which were apparently the most favorable to the production of a positive result in the mixtures of bacteria and serum, an *S. aureus* isolated from a negative serum failed to give a positive result, so that it is evident that certain strains of the same species differ in this respect.

The fact that under certain conditions such common bacteria as staphylococci and streptococci, when growing in normal serum, may give rise to a positive Wassermann reaction, is of the greatest practical importance, and one might conclude from these experiments that only fresh sera should be used in making complement fixation tests; but if proper precautions are taken in the collection of the specimens, there is no danger of bacterial contamination, and we believe that, unless such contamination occurs, a positive result will not be obtained in a normal serum. Our experiments have shown that sera may be kept at room temperature for as long as a month without danger of a negative serum becoming positive, and we have observed such a phenomenon only in sera contaminated with bacteria. Moreover, contaminated sera may be easily detected, as they appear cloudy and have a disagreeable odor.

However, these observations indicate the necessity of asepsis in the collection of blood for the Wassermann test, if the test can not be made within twenty-four hours after collection, and we believe that such precautions should always be taken in the collection of blood for this purpose. The needle or lancet used for puncture,

and the Wright tube in which the blood is collected, should be sterilized, and the skin over the site of puncture thoroughly washed with alcohol. When the blood is to be examined at once, such measures are unnecessary, but they should always be adopted if the serum is to be kept twenty-four hours or longer before making the test.

We believe that the results obtained in the experiments described justify the following conclusions:

1. Certain strains of staphylococci and streptococci, when growing under favorable conditions in normal human blood serum, are capable of producing substances in the serum which cause a positive result with the complement fixation test for lues.

2. These non-specific reactions do not occur in normal serum which is uncontaminated with bacteria, even though the serum be kept at room temperature for one month.

3. The intensity of the positive reaction in contaminated sera apparently depends upon the presence of favorable cultural conditions for the bacteria concerned, as contaminated sera in which the bacteria can not develop do not give a positive reaction.

4. When growing in normal blood serum certain strains of staphylococci and saprophytic bacilli produce thermostabile anticomplementary bodies which are capable of causing total inhibition of hemolysis in both the antigen and control tubes.

5. Non-specific reactions due to bacteria are probably very rare in practice, but they are of sufficient importance to justify the use of aseptic methods in the collection of blood for the complement fixation test.

STUDIES IN ISOAGGLUTINATION.

II. THE OCCURRENCE OF GROUPED ISOAGGLUTINATION IN THE LOWER ANIMALS.*

By REUBEN OTTENBERG and S. S. FRIEDMAN.

(From the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and the Pathological Laboratory of Mount Sinai Hospital, New York.)

In the previous paper of this series,[1] the isoagglutinins which occur in nearly all human bloods, and according to which all human beings can be divided into four sharply separated groups, were discussed. For some time it has seemed to us that a constant and hereditary characteristic like this grouping must have some very fundamental significance, and is not likely to be limited to one species of animal.

Throughout the literature it is stated that normal isoagglutinins occur only in human beings. Von Dungern has recently shown that immune isoagglutinins can be developed in dogs by treating them with each other's blood, and that two isoagglutinable substances occur in dog bloods. He has also shown, with the method of absorption of agglutinins from animal sera by human red blood cells, that the serum of many animals contains agglutinins which have the same selective activity on human cells as that shown by human isoagglutinins. Likewise he has demonstrated by the absorption method with human sera and the red cells of other animals, that the cells of many other animals contain agglutinable substances

* Received for publication, March 17, 1911. This study is one of a projected series on *isoagglutination*, which in turn constitutes a section of a comprehensive plan of research on the composition of protoplasm, as well as the structural and dynamic relationships of cell constituents and products. These investigations are now in progress in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and under the auspices of the George Crocker Special Research Fund.

[1] Ottenberg, *Jour. Exper. Med.*, 1911, xiii, 425.

identical in their susceptibilities with the isoagglutinable substances of human cells.

Grouped isoagglutination in animals seems to have escaped observation.

EXPERIMENTAL OBSERVATIONS.

We have investigated the occurrence of isoagglutination in thirty-two rabbits and in eleven steers.

Blood of Rabbits.—The rabbits were taken in groups of about ten, and the serum of each rabbit was tested against the red blood cells of all the other rabbits in the group. Suspensions (3 or 5 per cent.) of washed red blood cells in 0.9 per cent. salt solution were used; and in general one volume of cell suspension was mixed with three volumes of serum. The mixtures were made in capillary pipettes of three to five millimeters in diameter, using the modified Wright technique as described by Epstein and Ottenberg.[2]

In the first set of examinations of rabbits, the tubes were sealed with paraffin and kept at room temperature for twenty-four hours. In the remaining series, the tubes were put in a thermostat for one hour at 37° C., and then in an ice box for twenty-four hours. The results were about the same.

TABLE I.

Sera.

Cells			I			II			III			IV	Kind of rabbit.
			3	6	12	13	14	1	9	10	11	7	
	I	3	−	−	−	−	−	−	−	−	−	−	White
		6	−	−	−	−	−	−	−	−	−	−	White
		12	−	−	−	−	−	−	−	−	−	−	Hare
	II	13	+	++	+	−	−	−	−	−	−	−	Black
		14	+	++	+	−	−	+?	−	−	−	−	White
		1	−	+	−	−	−	−	−	−	−	−	White
	III	9	++	+	++	+	++	++	−	−	−	−	Black
		10	++	+	+	+?	+	+	−	−	−	−	White
		11	++	++	++	−	+++	+	−	−	−	−	Hare
	IV	7	−	−	−	−	−	−	−	−	−	−	White

In those tubes in which the more marked agglutinations occurred, the phenomenon could be observed after about twenty to thirty

[2] Ottenberg and Epstein, *Arch. Int. Med.*, 1909, iii, 467

minutes. In the majority of cases, agglutination was seen only when the tubes were reëxamined after twenty-four hours. Most of the tests were examined microscopically also, but only agglutination which could be seen with the naked eye was recorded. In general, the agglutination results were not as striking as those with human blood. Tables I and II give the data arranged in groups.

TABLE II.

Sera.

Cells			I			II	III			IV				Kind of rabbit
			25	31	37	22	35	21	29	24	26	28	40	
	I	25	−	−	−	−	−	−	−	−	−	−	−	White
		31	−	−	−	−	−	−	−	−	−	−	−	White
		37	−	−	−	−	−	−	−	−	−	−	−	Gray
	II	22	+	++	++	−	−	−	−	−	−	−	−	White
	III	35	+	++	+?	+?	−	−	−	+?	−	−	−	Gray
		21	+	++	+++	++	+?	−	+?	+?	−	−	−	White
		29	+?	++	++	+	−	−	−	−	−	+?	−	White
	IV	24	−	−	−	−	−	−	−	−	−	−	−	White
		26	−	−?	−	−	−	−	−	−	−	−	−	White
		28	−	−	−	−	−	−	−	−	−	−	−	White
		40	−	−	−	−	−	−	−	−	−	−	−	White

Each series of observations was repeated and substantially the same results were obtained.

It is seen that the bloods divide themselves naturally into four groups. In group I the serum is agglutinative toward all agglutinable cells, but the cells are non-agglutinable. In group II the serum agglutinates cells of group III, but the cells are agglutinable only by the serum of group I. In group III the serum is generally non-agglutinative; the cells are agglutinable by the sera of groups I and II. In group IV the serum is not agglutinative, and the cells are not agglutinable (like the embryonic state of human blood). There was no relation of the grouping to the race or color of the animals. It was probably an accident that all the animals of group IV were white.

It is clear that the facts can be explained by the assumption that there are two agglutinins (which we may designate x and y) and two agglutinable substances (X and Y). Group I possesses agglu-

tinins x and y, but no agglutinable substance. Group II possesses agglutinin y and agglutinable substance X. Group III possesses agglutinable substances X and Y, but no agglutinin (with possibly occasional exceptions). Group IV possesses neither agglutinable substance nor agglutinin.

Studies of the effects of dilutions were made with a number of the sera. In general they produced decided agglutination up to a dilution of 1 in 4 (when a quarter of the total volume of the mixture was 5 per cent. cell suspension), but perceptible agglutination was observed with several of them up to a dilution of 1 in 10. It is probable that because of their feeble action these agglutinins have been overlooked.

Blood of Steers.—The blood of fifteen steers was defibrinated. Four which showed some laking were rejected. The other eleven were tested for isoagglutination with the same technique that was used in the rabbit experiments. The resultant agglutinations were much heavier than those obtained with rabbit blood, in fact they were quite as striking as human isoagglutinations.

TABLE III.

Sera.

			I		II						III		
			6	11	2	3	5	8	10	12	7	9	13
Cells	I	6	−	−	−	−	−	−	−	−	−	−	−
		11	−	−	−	−	−	−	−	−	−	−	−
	II	2	++	++	−	−	−	−	−	−	−	−	−
		3	++	++	−	−	−	−	−	−	−	−	−
		5	+	+	−	−	−	−	−	−	−	−	−
		8	+	++	−	−	−	−	−	−	−	−	−
		10	+++	++	−	−	−	−	−	−	−	−	−
		12	++	+++	−	−	−	−	−	−	−	−	−
	III	7	−	−	−	−	−	−	−	−	−	−	−
		9	−	−	−	−	−	−	−	−	−	−	−
		13	−	−	−	−	−	−	−	−	−	−	−

When the results are arranged in tabular form, the bloods of the steers are seen to fall into three groups (table III). Group I is agglutinative, but not agglutinable. Group II is agglutinable, but not agglutinative. Group III is neither agglutinable nor agglutinative. The grouping of these eleven bloods can be explained by assum-

ing that there is one isoagglutinin and one isoagglutinable substance. Bloods of group I possess agglutinin, but no agglutinable substance. Bloods of group II contain agglutinable substance, but no agglutinin. Bloods of group III contain neither agglutinable substance nor agglutinin. It is possible that examination of a larger number of animals may reveal the existence of a second agglutinin and a second agglutinable substance.

CONCLUSION.

Grouped isoagglutination is not limited to man, but is much more widespread than has been hitherto suspected. It occurs in the bloods of steers and rabbits. It seems probable that it will be found to occur in the bloods of other animals. Just how many of the isoagglutinins and the isoagglutinable substances in different species are respectively identical is still to be determined. The work is being continued with other animals.

STUDIES IN ISOAGGLUTINATION.

III. TONICITY IN ISOHEMAGGLUTINATION.*

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In the introduction to the first paper in the present series,[1] an outline was given of the main facts concerning the isoagglutination of human bloods, and the division of human bloods into four sharply defined varieties or groups, according to the way in which they agglutinate each other. For the present purpose these groups may be briefly summarized as follows: In group I the serum agglutinates the cells of all other groups, but the cells are not agglutinable. In group II the serum agglutinates the cells of groups III and IV, and the cells are agglutinable by the sera of groups I and III. In group III the serum agglutinates the cells of groups II and IV, and the cells are agglutinable by sera of groups I and II. In group IV the serum is non-agglutinative, and the cells are agglutinable by sera of all other groups.

Three years ago Gay attempted to show that a relation existed between the resistance of human red blood cells to laking by hypotonic solutions and their isoagglutinative grouping. In an examination of twelve bloods he found that corpuscles not agglutinated by any sera (i. e., those belonging to group I) are more susceptible to laking by saline solutions of low percentage content than are

* Received for publication, March 17, 1911. This study is one of a projected series on *isoagglutination*, which in turn constitutes a section of a comprehensive plan of research on the composition of protoplasm, as well as the structural and dynamic relationships of cell constituents and products. These investigations are now in progress in the Laboratory of Biological Chemistry of Columbia University, at the College of Physicians and Surgeons, and under the auspices of the George Crocker Special Research Fund.

[1] Ottenberg, *Jour. Exper. Med.*, 1911, xiii, 425.

other corpuscles, and that definite tonicity groups exist corresponding to agglutination groups. He suggested that, as sera are assumed to be of the same tonicity as the contents of the red cells,[2] the differences in tonicity might be the cause of the agglutinations.

The hypertonicity of sera belonging to the first group would explain their power of agglutinating cells of the other groups. The hypertonicity of the serum of one of the agglutinable groups would explain its agglutination of the cells of the other group. But, as Gay himself points out, it would not explain the reverse interaction between the groups, which also occurs.

Agglutination groupings are permanent for the individual. They are hereditary. Isoagglutinative serum is active at a considerable dilution—generally up to about 1 in 32—with ordinary physiological saline solution. The resistance of human red cells is known to vary widely in disease and under special conditions, such as follow the ingestion of salts (Sutherland and McCay). These important facts led us to doubt whether so simple an explanation as Gay offers can account for the phenomena. We therefore determined to reëxamine the subject and repeat Gay's experiments.

The technique which we followed was practically identical with that of Gay. With a clean, dry, hollow needle, fifteen to twenty cubic centimeters of blood were obtained from an arm vein and defibrinated by a few glass beads in a perfectly clean bottle. (Gay used a rod instead of beads, and obtained a somewhat larger amount of blood.) After the cells had sedimented during six hours in an ice box, all the supernatant clear serum was pipetted off and the sedimented cells were used for the tonicity tests. Bloods of which the serum showed any trace of laking were discarded.

It is not necessary to describe here again the well-known method of making the agglutination tests and of arranging the bloods in their correct groups.

The resistance of the red blood cells was tested by Hamburger's method, as used by Gay. A series of salt solutions ranging in concentration from 0.38 per cent. to 0.57 per cent. of sodium chlorid was prepared. In the first experiments, the difference between the

[2] This assumption is not entirely warranted, as the work of Theobald Smith and others has shown.

successive concentrations was 0.03 per cent., as in Gay's work. Later it was made 0.02 per cent. All precautions were taken to make the solutions accurate and constant. Chemically pure sodium chlorid dried to constant weight was used, and fresh solutions were prepared for each new group of cases tested. Evaporation was carefully prevented.

In testing each blood for tonicity, a series of small test tubes was arranged, each tube containing five cubic centimeters of one of the graded salt solutions. The sedimented unwashed red cells were shaken, to insure uniform distribution, and 0.1 cubic centimeter of the suspension was added to each tube. The tubes were shaken and allowed to stand at room temperature for a short time. Readings were made then, and again after the tubes had been in an ice box for twelve hours.

The amount of hemolysis was estimated for each tube of the series by comparing its color with the colors of a standard series made up separately with each blood. This scale was prepared by laking 0.4 cubic centimeter of the sedimented red cells in twenty cubic centimeters of distilled water (the same ratio of blood as that in the tests). This was designated 100 per cent. hemolysis and dilutions of this were then made to represent 80, 70, 60, 40, 30, 20, 10, 5, and 2.5 per cent. hemolysis. Each of these solutions was put in a tube of the same caliber as that of the tubes used in the tests.

In order to facilitate our discussion, we reproduce Gay's[3] tables.

GAY'S RESULTS.

Non-Agglutinable Cells. Class I.

NaCl solution (%).	0.39	0.42	0.45	0.48	0.51	
	Amount of hemolysis (percentage).					
Blood No. 18	100	75	40	5	5	Aug. 26
	100	100	60	35	10	Sept. 24
" 22	100	90	75	50	2.5	Aug. 18
	100	40	15	5	2.5	Aug. 26
" 34	80	80	35	15	10	
" 35	95	50	15	10	5	
" 38	95	35	10	10	2.5	

[3] Gay, *Jour. Med. Research*, 1907–8, xvii, 330.

Agglutinable Cells. Class II.

NaCl solution (%).	0.39	0.42	0.45	0.48	0.51	
	Amount of hemolysis (percentage).					
Blood No. 17	90	65	25	15	5	Aug. 18
	90	50	15	5	2.5	Aug. 26
	60	65	15	5	5	Sept. 24
" 26	85	35	10	5	5	Aug. 18
	40	10	2.5	2.5	2.5	Aug. 26
	85	35	5	5	5	Sept. 24
" 30	70	70	10	5	2.5	
" 33	60	50	10	5	2.5	
" 37	70	45	25	5	0	

Agglutinable Cells. Class III.

NaCl solution (%).	0.39	0 42	0.45	0.48	0.51	
	Amount of hemolysis (percentage).					
Blood No. 23	65	25	5	5	5	Aug. 18
	80	15	5	5	2.5	Aug. 26
	50	40	10	5	0	Sept. 24
" 25	60	15	20	5	5	

Gay's conclusions from these results are as follows: "(1) That human corpuscles which are not agglutinated by foreign human sera are uniformly more susceptible to laking by salt solution of low percentage than are corpuscles which are agglutinated by foreign isosera. (2) That definite groups as regards resistance to salt solutions are present, which groups coincide with the groups classified according to agglutination. (3) That the resistance of a given blood does not change apparently within a month or more, and probably corresponds to the relatively unchanging relations manifested in isoagglutination."

Gay's tables do not entirely justify his conclusions, as an examination of the data will show. Thus the first two of five bloods belonging to class II exhibited, on several occasions, only the most minute differences from the last three of the five in class I. The differences between the same blood on several dates are, in some cases (e. g., No. 26), as great as the differences between bloods in the different classes.[4]

Our own work consisted of the examination of the blood of twenty-two persons. In our first group of six cases (table I), the

[4] Relations must be ascertained from general comparisons of the results for one blood with the results for another blood. The irregularities are so numerous that a comparison of different bloods as tested by any particular salt solution involves contradictions. As a rule, readings of more than 70 per cent. hemolysis can not be accurate, because the intensity of the colors makes such readings extremely uncertain. These remarks apply to our own, as well as to Gay's results.

TABLE I.

Sodium chlorid solution (per cent.).	0.42	0.45	0.48	0.51	0.54	0.57
	Amount of hemolysis (per cent.).					
Group I	25	5	0	0	0	0
	20	2.5	0	0	0	0
	40	5	0	0	0	0
Group II	60	10	2.5	0	0	0
	60	7	2.5	0	0	0
Group III	50	15	2.5	0	0	0

three classes showed a definite grouping as regards corpuscular tonicity. The blood in class I, however, proved to be the most resistant, that is, it was the least hemolyzable by hypotonic saline solution. This, strangely enough, is exactly the reverse of Gay's finding. The two groups of agglutinable cells showed little difference in saline concentration from each other, those of class II being least resistant.

Our second series (table II) consisted of the bloods of six other individuals. Here, the blood of the first group (only one individual in this group) is most easily hemolyzed, and the agglutinable cells evidence most resistance.

TABLE II.

Sodium chlorid solution (per cent.).	0.40	0.42	0.44	0.46	0.48
	Amount of hemolysis (per cent.)				
Group I	80	50	20	10	2.5
Group II	50	40	20	15	5
	50	20	10	5	2.5
	80	60	20	5	0
	60	10	10	2.5	0
Group III	60	25	15	2.5	2.5

In our third group of ten cases (table III), the first or non-agglutinable group showed instances of both low and high tonicity. Here, the second and third groups showed high tonicity, the reverse of Gay's findings.

TABLE III.

Sodium chlorid solution (per cent.).	0.38	0.40	0.42	0.44	0.46
	Amount of hemolysis (per cent.).				
Group I	60	40	20	10	0
	50	40	15	5	0
	40	30	10	0	0
	80	60	20	5	0
	80	60	40	10	5
	80	70	40	10	5
Group II	70	60	30	15	5
	80	40	40	10	5
	90	80	60	20	10
Group III	90	80	60	40	20

Correlating our results, we see that instances of high and of low tonicity occur with about equal frequency in the groups of agglutinable and non-agglutinable cells. We therefore conclude that there is no support for the opinion that isoagglutination of human blood is due simply to variations of molecular concentration.

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THE DISTRIBUTION OF SOLUTIONS IN CARDIECTOMIZED FROGS.*

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INTRODUCTION.

Life depends upon a proper distribution of solutions throughout the animal body. This distribution is accomplished by the cardiovascular mechanism. Cardiac activity is therefore inseparably associated in our minds with life and all its manifestations. The permanent stoppage of the heart is the paramount sign of death. When the circulation ceases in a part of the body, that part is dead; it becomes necrotic. And if it happens now and then, that evidence of distribution appears in a part, the known blood-vessels of which were occluded by thrombi or emboli, or experimentally by ligation, we are sure that this has been accomplished by the ubiquitous collateral circulation. The marvelous efficiency of the mechanism of circulation is actually so great and so complete, that the possibility of the existence of other independent mechanisms for the purpose of distribution is rarely thought of. The activity of the migrating phagocytes can not be considered as such an independent mechanism. The value of the ameboid movements of these cells consists essentially in their ability to pick up substances; their actual locomotive power is comparatively insignificant. Their importance as distributing agents the leucocytes derive essentially again from the circulation which conveys them rapidly to distant parts of the body. The discovery of migration of tetanus toxin along the nerve fibers[1] seemed, indeed, to present such an independent mode of distribution; hence the interest that it aroused. But here again we find the wide-spread tendency among various investigators to assume that this migration takes place within the lymphatics of the nerve fibers;

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[1] Meyer and Ransom, *Arch. f. exper. Path. u. Pharm.*, 1903, xlix, 389.

in other words, again through a part of the circulatory system. The assumption that this system is the only mechanism existing for the distribution in the interior of the body, seems also to preclude the idea that adjoining sections of the body may benefit from local injections; for each injected solution has first to be conveyed to the heart in order to be distributed to all parts of the body alike. It was on this basis that Filehne[2] ridiculed the belief of some oculists, that an injection of strychnin in the temple benefits first the vision of the eye of the corresponding side.

While studying the question of migration of adrenalin along the nerves[3] I came across certain phenomena which indicated the possibility of a distribution of solutions in the body even in the absence of the cardiovascular apparatus. This started me on a systematic study of the present problem, that is, the distribution of solutions after eliminating the heart's action. I turned my attention to substances which are capable of bringing out well defined vital reactions. Under these circumstances, I had, of course, to study this problem on animals, the tissues of which survive for some time the elimination of the circulation, i. e., on frogs. Various substances were studied. In this preliminary communication, however, I shall report briefly the results obtained with three substances, the action of each of which represents a separate type of effects. These substances are: adrenalin, strychnin, and morphin.

Method.—The frogs used were *Rana pipiens* and *Rana clamitans*, the species usually employed in American laboratories. It is necessary to mention this, because various species respond in different ways to some alkaloids, strychnin[4] for instance. Our method was simple. After exposing the heart under ether anesthesia, a ligature was firmly applied above the auricle, and the ventricle was cut off; the abdominal incision was then closed. This method has the disadvantage, that, when injections are made into the abdominal cavity, some of the injected fluid may escape through the wound. In female frogs the eggs in conjunction with the large liver seem capable of completely preventing any escape of the fluid. In experiments in which an escape of the solution might throw doubt upon the validity of the result, in adrenalin experiments, for instance, the conclusions were drawn chiefly from injections made into other parts of the body. In another method, the abdomen was not opened, but with a curved needle, thrust through the intact abdominal wall, two ligatures were

[2] Filehne, *Arch. f. d. ges. Physiol.*, 1901, lxxxiii, 380.
[3] Meltzer, *Arch. f. exper. Path. u. Pharm.*, 1908, lix, 458.
[4] See Poulsson, *Arch. f. exper. Path. u. Pharm.*, 1889, xxvi, 22.

firmly applied around the heart, one just below the clavicles and the other about eight millimeters posteriorly to the first. This method leaves the abdominal cavity intact, and furnishes, as a rule, very good results. In some instances, however, the upper vessels of the heart were missed by the ligature, and at autopsy the heart was found still beating. Our conclusions, therefore, were established in the first place by the first mentioned method, in which all circulation was definitely eliminated.

The use of ether in these experiments presents in some instances a disturbing factor, since after removal of the heart the animal can not get rid of the ether through the lungs in the usual manner. This is a disturbing factor in judging the onset of a depression or an excitation. In a number of experiments, instead of using ether, the brain of the animal was destroyed previous to the exposure of the heart. The inhibitory effect of this operation upon phenomena depending upon the normal excitability of the spinal cord, was met by permitting a long interval to elapse between this operation and the exposure of the heart. However, while this method was satisfactory as far as excitation phenomena were concerned, it failed completely when the onset of the depressing action of some substances had to be considered. I could not make up my mind to expose the heart without the use of ether, although the experiment evidently requires it. I tried to meet the difficulty by operating as quickly as possible and thus reducing the amount of the anesthetic given. Then in experiments in which the depressive action has to be observed, some time was permitted to pass after the removal of the heart before an injection was given.

It is an interesting fact that these animals *do* awake from the ether anesthesia. This was especially evident when the anesthesia was somewhat prolonged. Soon after the animal is removed from the board and put under an open glass bell, it may even stay on its back for a while or turn over and sluggishly pull up its legs, and remain sitting on the same spot with its head resting on the table. Gradually, however, it will wake up and begin to jump around or against the transparent glass wall; it attempts to escape with the dexterity of a normal animal, as soon as it discovers an opportunity; in short, it acts like a normal frog. Apparently the ether escapes gradually through the skin, which it has to reach, not as in normal animals by the convenient road of the circulation, but by direct penetration of all the intervening tissues.

EXPERIMENTAL RESULTS.

Adrenalin.—A few years ago we[5] found that an injection of adrenalin into a lymph sac of a frog will cause a characteristic

[5] S. J. Meltzer and Clara M. Auer, *Am. Jour. Physiol.*, 1904, xi, 28, 37, 40.

maximal dilatation of the pupils. Furthermore, also in an enucleated bulbus of a frog, when bathed with adrenalin, the pupil will dilate promptly. Finally such a bulbus responds readily with this reaction to adrenalin for many hours after the enucleation. In this dilatation of the pupil I had then a sharp vital reaction to adrenalin, which is capable of surviving the removal of the heart for many hours. I began, therefore, the investigation of the problem in hand by studying the effect of injections of adrenalin into cardiectomized frogs upon the subsequent condition of their pupils.

Results.—Many varied experiments were performed, but I shall give here only the general results, which are as follows: When one cubic centimeter of adrenalin in the usual dilution (1:1000) was injected into the dorsal lymph sac of a cardiectomized frog of medium size, sooner or later both pupils became dilated. The dilatation may have begun about thirty to forty minutes after the injection and reached the maximum in sixty to ninety minutes. The onset and development of the dilatation may have been considerably retarded in one or the other case, depending upon a variety of conditions, but it has never failed entirely to make its final appearance. As a rule, the dilatation occurred in both eyes simultaneously; but there were some exceptions. The injection into the dorsal sac was given usually through the muscles of one thigh, for the purpose of preventing the escape of the solution. It then happened, sometimes, that the pupil of the eye on the side through which the adrenalin was injected, became dilated before the pupil of the other eye. When the injection was given into the *lateral* lymph sac, this preference of one pupil was the rule, that is, the pupil of the eye on the side into which the injection was given became dilated first; the dilatation of the pupil of the opposite side followed perceptibly later. When the injection was given into the abdominal cavity, the dilatation of the pupils began to appear sometimes as late as two hours after the injection. If the injection into the cavity was given through one of the flanks, again the dilatation usually appeared first in the pupil of the eye of the corresponding side.

Injections into the legs brought, as a rule, late dilatations of the pupils. But in the cases in which only one cubic centimeter was administered into one of the legs, there were a few instances in

which only a slight dilatation, or even no dilatation at all, made its appearance. In the vast majority of the cases, however, there was a definite, even a maximal, dilatation. But in most of these cases the pupil of one eye was more favored than the other, and it sometimes happened that the pupil of the eye on the opposite side of the injected leg was the favored one. When one cubic centimeter of adrenalin was injected into each, the dilatation set in a good deal earlier, appeared always in both pupils simultaneously, and was generally maximal.

The dilatation of the pupils was not interfered with by the section of one or both of the sciatic plexuses, which proves that the forward migration of the adrenalin did not take place along the nerve fibers.

The dilatation did not fail to appear even when the frog was suspended by the head; the distribution then is accomplished even against gravity. However, gravity is apparently capable of exerting a favorable influence upon the process of migration, for in all instances in which the head of the animal was kept lower than the body, the dilatation set in unmistakably earlier.

In a few instances, cardiectomized frogs were kept wrapped in moist filter paper for twenty-four hours and longer, at a low temperature in the refrigerator. Even the pupils of the eyes of these animals seemed sometimes to be capable of still responding moderately to an injection of adrenalin. Here, however, another device proved to be much more efficient. After enucleation of the eyes of these animals, fresh bulbi from normal animals were inserted into the orbits, the anterior surface of the bulbus touching the inside of the orbit. Two hours after an intra-abdominal injection of adrenalin into the cardiectomized frogs from the refrigerator, the pupils of these alien eyes became dilated as if they had been directly bathed with adrenalin. Furthermore, when these bulbi were removed and substituted by new fresh eyes, the pupils of these eyes, too, became dilated in less than an hour. This shows that there was in the orbit a quantity of adrenalin sufficient to dilate the pupils of several eyes.

It should be mentioned that in many of the foregoing experi-

ments the dilated pupils of the cardiectomized frogs became again gradually smaller.

Besides the dilatation of the pupil, an injection of adrenalin into a normal frog causes also a secretion of mucus from the skin (Ehrmann).[6] In my experiments, when an injection of adrenalin was given into the dorsal lymph sac of freshly cardiectomized frogs, a definite secretion of mucus began to take place, in spots, on the back and to the side of the animal, soon after the injection. The secretion seemed to spread forward from the posterior end. In frogs which were kept in the refrigerator for twenty-four hours, an injection of adrenalin did not produce such a secretion. The secretion of the freshly cardiectomized animals was apparently produced by the stimulation of the still living secreting cells of the skin by the adrenalin, while spreading through the lymph sacs. After twenty-four hours, these secreting cells are apparently dead and do not respond to such stimulation.

These experiments brought definite evidence that even after complete exclusion of the cardiovascular mechanism, adrenalin is capable of spreading through the entire body of the animal in a comparatively short time, causing specific vital reactions of tissues with which it comes in contact while wandering through the body. In passing from one lateral lymph sac to the iris of the eye on the opposite side, or in passing from the abdominal cavity to either of the eyes, the adrenalin has to pass through various solid membranes and voluminous tissues. Surely it could not pass them by diffusion alone. It migrates against gravity. It migrates in an animal in which there is no sign of movements capable of assisting in the forward movement. In animals which have been in the refrigerator for forty-eight hours, there are surely no ciliary movements or other cell activity to help along the wandering solution.

It will not be amiss to state here that the removal of the heart means, indeed, the complete removal of the entire cardiovascular system. The lymphatics help the distribution by emptying their contents into the veins. The same is the case with the lymph hearts; they take up lymph during their diastole and empty it into a vein during the systole. Lymph hearts can not assist in the dis-

[6] Ehrmann, *Arch. f. exper. Path. u. Pharm.*, 1905, liii, 137.

tribution of solutions, except as aids to the cardiac circulation. When the heart is ligated and cut out, the activity of the cardio-vascular apparatus, including lymphatics and lymph hearts, is completely eliminated.

Strychnin.—After the experience with the effect of adrenalin upon the iris, a tissue which survives the cardiectomy for a good many hours, I began to study the effect of substances which are capable of bringing out well defined reactions by their stimulation of the central nervous system, especially the spinal cord. This organ evidently loses its irritability only gradually after the elimination of the cardiac circulation. As stated already, immediately after cardiectomy, i. e., when the animal is out of the effects of ether, it jumps and acts like a normal frog. Soon, however, it becomes quiet and does not jump away when the opportunity presents itself. It still holds its head up and turns over rapidly when put on its back. Gradually the head goes down on the table, the movements become sluggish, the legs are pulled in only half way, and the animal makes an unsuccessful attempt to turn over when put on its back. Soon after this, no attempt is made, the animal responding sluggishly to stimulation, the legs remaining in any position in which they are put, and no sign of a voluntary motion can be observed. Finally also, all signs of reflex irritability disappear. The duration of the survival period varies with the temperature; at a higher room temperature the end might come after thirty minutes, at low temperature, however, reflex irritability may survive the cardiectomy for 150 minutes. I may say positively that in these control animals with mere cardiectomy, there was at no time a sign of increased reflex irritability—unless, in addition, the brain was destroyed.

Strychnin is, of course, the most appropriate substance for bringing out unmistakable signs of a stimulating action upon the cord. A number of different experiments were made with this substance, of which I shall record here only the general results.

An injection of one or two milligrams of strychnin in any part of the body of a medium sized freshly cardiectomized frog does not fail to bring out a characteristic hyperexcitability. In many instances this may begin to show twenty or thirty minutes after

the injection. It begins with a slight hyperesthesia, which increases gradually to a state in which each touch brings out a tetanic response, until finally the animal is lying continuously stretched out, having very frequent spontaneous violent tetanic attacks, lasting many seconds. Gradually the duration and the frequency of the attacks diminish until the animal dies. Without exception, animals receiving moderate doses of strychnin survive the controls by one or two hours, or even longer.

When large doses of strychnin are injected, for instance, twelve or fifteen milligrams, one of two things may happen. Either the cardiectomized frog gets spontaneously a very violent tetanus within a few minutes after the injection; in this case, which usually occurs when the strychnin is given into the dorsal lymph sac, the tetanic attacks become rapidly weaker and the animal becomes progressively paralyzed. Or, very soon after the injection, the animal becomes strikingly depressed and semiparalyzed; gradually however, definite but ineffective signs of increased reflex irritability become manifest, breaking inefficiently through the paralysis of the animal. This form of effect occurs, as a rule, when the injection was made into the abdomen. In either case, however, these animals die invariably sooner than the control frogs.

In animals of the second class, which usually received the strychnin intra-abdominally, it happens that some of the strychnin escapes through the sewed-up incision. In one such instance, a control frog was placed near the strychnin animal; after a while we were confronted with this remarkable sight: the control frog was having violent tetanic attacks, while the animal with the large doses of strychnin in its abdomen was paralyzed and dying. The explanation is that the escape of strychnin on the table entered through the wound of the control animal in a quantity sufficient to produce tetanus but not enough to cause paralysis.

We thus see that in cardiectomized frogs strychnin is capable of producing, by smaller doses, a well defined tetanus, and by larger doses also, or predominantly, a paralysis, exactly as in frogs with a normal circulation, except that in the cardiectomized frogs larger doses have to be administered.

That the paralysis produced by strychnin is of central origin, and not due to fatigue of the muscles from the preceding tetanus, is here shown better than in the normal frogs by such cases in

which the paralysis predominated from the start, and in which at no time a real tetanus occurred which could cause fatigue.

These experiments furthermore effectively dispose of the theory of Verworn[7] regarding the nature of the strychnin paralysis. Verworn and his pupils maintain that the paralysis is not due to a primary action of strychnin upon the nerve cells of the cord, but is due only to asphyxia which is being brought about by the paralysing action of strychnin upon the heart. This view of Verworn caused a great deal of discussion. The experiments on the cardiectomized frogs show conclusively that moderate doses of strychnin are capable of causing prolonged violent attacks of tetanus in the complete absence of any circulation, and that only large doses of strychnin cause paralysis. The paralysis, therefore, can not be due to the absence or paralysis of the heart; it is due to a direct paralysing action of strychnin upon the nerve cells.

The action of strychnin upon the motor nerve endings (Richet, Vulpian) has not yet been sufficiently studied to permit here a definite statement on that subject.[8]

Morphin.—The observations made with adrenalin and strychnin show that these substances become distributed throughout the body of the cardiectomized frogs and that they produce qualitatively in these animals the same kind of effects that they produce in animals with a normal circulation. The experiments with morphin, however, revealed a remarkable aspect of the action of some substances upon cardiectomized frogs. When ten to fifteen milligrams of morphin are introduced into any part of a normal frog and the animal is observed for hours, no definite effect can be noticed. After two or three days, however, such an animal may develop a definite tetanus. However, when a dose of only five or six milligrams is injected into the lymph sacs or the abdominal cavity of a cardiectomized frog of medium size, the animal shows definite tetanic

[7] Verworn, *Arch. f. Physiol.*, 1900, 385.

[8] Prof. W. B. Cannon called my attention to a passage in Vulpian's "Leçons sur l'action physiologique des substances toxiques et médicamenteuses," Paris, 1881, p. 46, in which it is stated that Stilling, in 1842, "a montré que l'on peut obtenir les effets caractéristiques du strychnisme, en introduisant de la strychnine sous la peau d'une grenouille dont tous les viscères, le cœur y compris, ont été enlevés."

attacks thirty or forty minutes after the injection. After doses of ten or fifteen milligrams, the animal develops at first a depression and considerable relaxation; the animal may come to lie on its abdomen with its head on the table and its legs relaxed and in any position. When turned on its back, it may remain in this position without even attempting to turn over. Suddenly the animal seems to assert itself. It turns back with great rapidity, it jumps away when it is touched, otherwise it sits on one spot with its legs pulled in and its head raised. Gradually a strong hyperesthesia develops which terminates with complete tetanus. Such morphinized animals can not be distinguished from frogs which received strychnin. When a cardiectomized frog of medium size receives about thirty milligrams of morphin, a strong depression and a semiparalysis develop early from which the animal is practically unable to recover; but this paralysis does not conceal completely the development of a hyperexcitability. During this state a touch of the prostrated animal may bring out suddenly a brisk short movement of one hind leg or of both, which result, however, can not be obtained again for a minute or two. Such repeated stimulations seem to hasten the death of the animals.

We see here, then, the remarkable fact that in a frog without circulation tetanus may be brought on by morphin incomparably more rapidly and with greater effect than in a frog with normal circulation, and this even with a smaller dose. Furthermore, a dose of morphin which causes practically no depressing effect upon normal frogs produces rapidly a profound depression and semiparalysis upon cardiectomized animals. In other words, the distributing mechanism of the cardiectomized animal acts in the case of morphin more promptly and efficiently than the cardiovascular mechanism. This is surely an unexpected and most remarkable result.

The most plausible explanation of this surprising fact seems to me to be that the normal circulation receives a variety of secretions from all parts of the body, which are capable of neutralizing, modifying, and retarding the effects of any single substance taken up by the circulation. In the absence of the centralizing activity of the cardiac circulation, the specific action of the injection substances which are now being distributed by some peripheral mechanism is

not being interfered with. In the case of morphin, we may assume that the circulating blood contains a secretion from one organ or from several organs, which is capable of modifying its actions upon the central nervous system. In the absence of the circulation, the organs in question stop their secretion and the morphin is permitted to display its specific action without outside interference. This hypothesis has the advantage of being amenable to a test.

DISCUSSION.

The foregoing experiments bring out two facts of a general character: (1) Animals possess a mechanism which is capable of distributing soluble substances through the entire body in the complete absence of circulation. (2) Some substances, when distributed by this mechanism, act even more rapidly and efficiently than when distributed by the cardiovascular mechanism.

In contradistinction to the centralizing cardiovascular apparatus, I shall designate the mechanism which attends to the distribution in cardiectomized animals as a peripheral one. I shall not enter into a particular discussion of the forces which are active in the process of distribution by this mechanism. I have already pointed out that gravity can not play an essential part in it. The movements which zoologists make responsible for the distribution of the mesolymph in the lower animals without a circulatory apparatus can not be a factor in our mechanism, as the distribution takes place also in perfectly motionless animals. Neither can ciliary or other cell activities have anything to do with migration of the solutions, since the distribution is efficiently accomplished also in animals dead and refrigerated for forty-eight hours. The forces which are concerned in the distribution are probably diffusion and osmosis and perhaps also imbibition, capillarity, surface tension, or even chemotaxis.

More important, for the present, is the question of the paths by which the peripheral distribution takes place. When the heart is ligated and excised, all motion in the arteries, capillaries, and veins stops, and none of the contents of the veins can pass into the arteries and capillaries and thus to distant tissues. As to the lymphatics, their function consists in carrying the lymph from the tissues to the veins. Now since the veins can not carry the lymph any further, it

is evident that in cardiectomized animals the lymphatics can render no service in the process of distribution of tissue lymph. By what path, then, is the injected fluid distributed in the cardiectomized animals? For those who have learned to distinguish between the lymphatics and lymph spaces, it is evident that in animals without circulation the distribution can take place only through the lymph spaces. However, self-evident as this distinction may be, that conception has not yet become common property. Fifteen years ago we[9] called attention to the incongruities which followed from "the general practice of confounding the lymph spaces with the lymphatics." "The so-called lymph spaces are those . . . interstices of all kinds naturally present between the elements or group of elements of the tissues of the entire body. They are of variable shape and size." ". . . all these spaces are more or less completely connected throughout the entire body and present a unity. . . ." We brought forward reasons "for considering the lymph spaces as an independent system. . . ." We tried to show that the nature of the fluid in the lymphatics differs essentially from that which is present in the lymph spaces. We therefore discarded the terms "lymph spaces" and "lymph" and suggested instead the terms "instertitial spaces" and "tissue fluid."

I shall now adopt the term "tissue spaces," which is used by some histologists and physiologists. In the past fifteen years, a great deal of histological and embryological work has been done which establishes clearly the difference between lymphatics and tissue spaces histologically as well as genetically. I need only refer to the valuable work done in this country by Mall and his colaborers, by MacCallum, and by Huntington. Fascinating are the important investigations of Sabin, which show the venous origin of the lymphatics. Physiologically, however, no progress has been made in that direction and we are still confronted with statements in the literature of physiology or experimental pathology, or made in public discussions, which indicate that the fundamental distinction between lymphatics and tissue spaces has not yet become general.

In the present series of experiments we brought forward unmis-

[9] Adler and Meltzer, *Jour. Exper. Med.*, 1896, i, 512.

takable physiological evidence that solutions may spread and spread even fairly rapidly and effectively through an animal body which has been deprived of all circulation. This distribution of solutions in such an animal can be accomplished only by way of the tissue spaces. This is a fair physiological proof of our contention that the tissue spaces present a unity, an independent system.

It seems to me that this system of tissue spaces, which serves as a basis for the peripheral mechanism of distribution in animals after they are deprived of their circulation, serves probably also as a distributing system in living animals which do not possess a circulatory apparatus. The peripheral mechanism might therefore possess phylogenetic significance, and I may, perhaps, be permitted to express the meaning of my observations in the following manner: animals which gradually acquire a complete centralizing circulatory apparatus never lose completely the primitive mechanism for distribution; during normal life it is overshadowed by the all-powerful central apparatus and becomes manifest only in the complete absence of the latter, but perhaps also when the efficiency of the central mechanism is only reduced.

The contrast between the circulatory apparatus and the peripheral mechanism, which has been touched upon before, ought to be especially emphasized. The circulatory apparatus distributes with great rapidity all specific secretions and juices from every part of the body to every other part. It thus neutralizes many antagonisms and reduces greatly the specificity of the activities of the various organs. It unifies all specific activities for one common purpose: the welfare of the sum of all the organs and tissues. The cardio-vascular circulation is a strong centralizing government which keeps down as much as possible the autonomous tendencies of the individual organs and parts of the body. The mechanism of distribution by way of the tissue spaces is far from being capable of exercising such a central power. By the virtue of the slowness of the distribution and by the fact of not possessing private roads for it, it is able in a very small degree only to unify the activities of the various organs. I have already designated the *mechanism of distribution by the tissue spaces as peripheral,* in the first place on account of the contrast to the central circulation. I may add now

that the distribution occurs apparently essentially through the lymph spaces of the peripheral parts of the body. On the basis of the foregoing analysis, we may now say that the peripheral mechanism, in contrast to the centralizing circulation, favors, in the first place, the *autonomous activities of organs,* that is, one organ may develop its activities to a greater degree than is allowed under the rule of the central circulation, without interfering or being interfered with too much by other organs. In the second place, it *favors local action,* i. e., a secretion or an injection into one territory may affect this or a neighboring territory to a much greater degree than distant territories; for instance, a limited quantity of adrenalin injected into a lateral lymph sac may affect the pupil on the corresponding side not only sooner, but also to a greater extent, and may perhaps even be confined exclusively to this side.

Our experiments so far have actually demonstrated only the existence of such a peripheral mechanism which favors autonomous action and local effects only in the complete absence of the central circulation. Can it be active also in the presence of the circulation? So far we have no evidence which will permit us to answer this question affirmatively in a positive manner. But we may claim that such an assumption is at least permissible. Assumptions to that effect may be made in several ways. In the first place it may be assumed that the peripheral mechanism is practically in action to some degree in all normal instances, that is, the central circulation does not suppress completely the autonomous and local activity of the peripheral mechanism. A closer experimental study of the subject may reveal this vestige of activity. It may, for instance, be correct that an injection of strychnin in the temple will favor, in the first place, the vision of the eye on the same side, just as the adrenalin in cardiectomized frogs favors the pupil next to it. Filehne's negative view came from his failure to distinguish between lymphatics and lymph spaces (tissue spaces).

In the second place, the peripheral mechanism may be normally active to a somewhat greater extent in all parts in which the circulation is normally not extensive. I need not speak of the cornea which has no circulation at all; there the distribution occurs exclusively by the peripheral mechanism, that is, through the lymph

spaces. I have in mind rather the central nervous system, which, according to the best anatomical authorities, has no lymphatics, and where also the normal exchange of fluid material through the blood capillaries appears to be restricted, at least in comparison with the exchange in other tissues. Here the *peripheral mechanism may be normally active and may be the means of favoring autonomous and local activities of the central nervous system.* A study of the various phases of the activities of the central nervous system from this point of view may perhaps be the means of throwing new light on some old questions.

I have also in mind the migration of tetanus and diphtheria toxin along the nerves. The nerves are only poorly provided with blood-vessels and lymphatics. The peripheral mechanism, i. e., the transmission of fluid through the tissue spaces of nerves may perhaps be normally an active agent. The migration of tetanus toxin through the axis cylinder means here migration through tissue spaces and is in a sense not specific. In the discussions of this subject, frequently no distinction has been made between lymphatics and lymph spaces.

Furthermore, the peripheral mechanism might become prominently active when, under pathological conditions, the circulation is abolished or reduced in a part of the body. Here we may think of the conditions which follow the slow occlusion of a vessel by a thrombus and perhaps also the rapid occlusion by an embolus. It is possible that under these conditions the peripheral mechanism takes an active share in the necessary local exchange of lymph between the devascularized area and the surrounding normal territories, at least during the transitional period before the development of the collateral circulation, and perhaps also during the establishment of an insufficient collateral circulation. It will have to be a matter of future investigation how much of that which is currently ascribed to the activity of a collateral circulation is actually due exclusively to a central mechanism and not to the action of the peripheral mechanism.

Finally, the service of the peripheral mechanism may be called into play also when the general circulation, for one reason or another, becomes inefficient; the excess of lymph which is not

carried off by the blood capillaries and the lymphatics is then carried further through the system of tissue spaces. When the impediment of the circulation is too great and the system of tissue spaces becomes overloaded with lymph, we have then the picture of general edema and anasarca.

SUMMARY.

Strychnin and adrenalin when injected into cardiectomized frogs are efficiently distributed all over the animal body and when administered in sufficient quantities produce the usual reactions of these alkaloids.

The experiments with strychnin prove positively that the paralysing effect of this poison is due to its direct action upon the nerve cells of the cord, and not, as Verworn assumes, to a paralysing action upon the heart.

When morphin is injected into cardiectomized frogs the effects are much greater and incomparably more rapid than when administered to a normal frog. The normal circulation probably contains substances derived from some organs capable of modifying and retarding the specific effects of morphin.

The distribution of solutions in animals deprived of their circulatory apparatus takes place through the tissue spaces, which present a more or less well connected system of communications throughout the entire animal body, especially through its peripheral parts. *This mode of distribution is designated as the peripheral mechanism.* In contrast to the centralizing cardiovascular apparatus, *the peripheral mechanism permits a greater autonomous action of organs and a more localizing effect of injections.*

The peripheral mechanism found to be active in animals deprived of their circulation is probably identical with the mechanism which serves for distribution of the mesolymph in living animals not yet possessing a circulatory apparatus. The existence of the peripheral mechanism in animals with a cardiovascular apparatus possesses probably a phylogenetic significance.

It is assumed that the activity of the peripheral mechanism is probably not completely suppressed even in the presence of a normally acting circulatory apparatus; that it exerts a greater physio-

logical influence in parts in which the circulation is normally somewhat difficult; and that it unfolds an activity in pathological conditions in which the circulation has been eliminated or reduced in some parts of the body, or in which the energy of the entire circulation has been reduced.

THE INFLUENCE OF DISTILLED WATER ON THE HEALING OF SKIN WOUNDS IN THE FROG.*

By EDWARD S. RUTH, M.D.

(*From the Laboratories of the Rockefeller Institute for Medical Research, New York.*)

PLATES LXXIII AND LXXIV.

It has been established by Jacques Loeb for certain marine animals that growth is accelerated by a slight diminution and retarded by a slight increase in the osmotic tension of the surrounding fluid medium. In this article it is my purpose to describe briefly the effects of variations of the dilution of the culture media and of the blood and lymph upon the healing of cutaneous wounds *in vitro* and *in vivo*.

THE EFFECTS OF DISTILLED WATER IN VITRO.

The epithelial proliferation of the skin of the adult frog is markedly increased by diluting the plasma with distilled water. Small fragments of skin have been cultivated in plasma containing one fifth, one fourth, two fifths, one half, three fifths, three fourths, and four fifths distilled water, and it has been noted that the increase of proliferation is already apparent in the one fifth dilution, and reaches its maximum in the one half dilution. When the dilution is carried further, the rate of proliferation progressively diminishes, until with plasma containing three fourths and four fifths distilled water, it ceases entirely (chart 1). Hence it follows that the growth of the cutaneous epithelium of the adult frog is influenced by the degree of dilution of the medium, as are certain other tissues.[1]

The influence of a diluted medium on the healing of cutaneous wounds *in vitro* of the skin of the frog was next determined. A method analogous to the one already described was employed.[2]

* Received for publication, March 20, 1911.

[1] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 562.

[2] Ruth, *Jour. Exper. Med.*, 1911, xiii, 422.

Wounds of equal size were made in the skin of the back with a small steel tube with a cutting edge, after which small fragments of skin containing the wounds were resected and implanted in normal and diluted plasma. The growth of the epithelium from the edges of the wounds toward the center could be followed under the microscope and drawn with the camera lucida. An accurate comparison of the rate of growth in normal and diluted plasma was easily secured. The earliest indication of repair by epithelial proliferation was observed in the plasma diluted one half with distilled water. In one experiment the epithelium was observed to begin to wander from the edges of the wound after five hours in this diluted plasma, while in normal plasma the wandering was observed only after fourteen hours. Moreover, the cicatrization was completed earlier in the plasma containing one half distilled water and was retarded when less than one half was used. The appended camera lucida drawings (figure 1) exhibit the progress of the cicatrization and show unmistakedly the accelerating effect resulting from the dilution of the plasma.

THE EFFECTS OF DISTILLED WATER IN VIVO.

In order to secure dilutions of the blood and lymph within the body of the frog, distilled water was injected into the peritoneal cavity. Then wounds of equal size were made in the skin with the cutting tube, and the rate of cicatrization was observed. The wounds were identical in the normal control and in the frogs injected with distilled water in quantities of two, three, five, seven, and ten cubic centimeters respectively. Still another series of frogs was injected, each with one cubic centimeter of sodium chlorid solution of the following strengths: 0.015, 0.02, 0.03, 0.06, 0.07, and 0.10 gram per cubic centimeter. The wounds and surrounding fragments of skin were extirpated after eighteen, twenty, and twenty-two hours, and subjected to microscopical examination.

The results were striking. The appearance of the wounds was almost identical with those undergoing cicatrization in cultures, and the newly proliferated epithelium was readily seen and drawn. The healing of the wounds of the frogs injected with hypertonic salt solution was retarded, or had not even begun, while in the control animals it was complete.

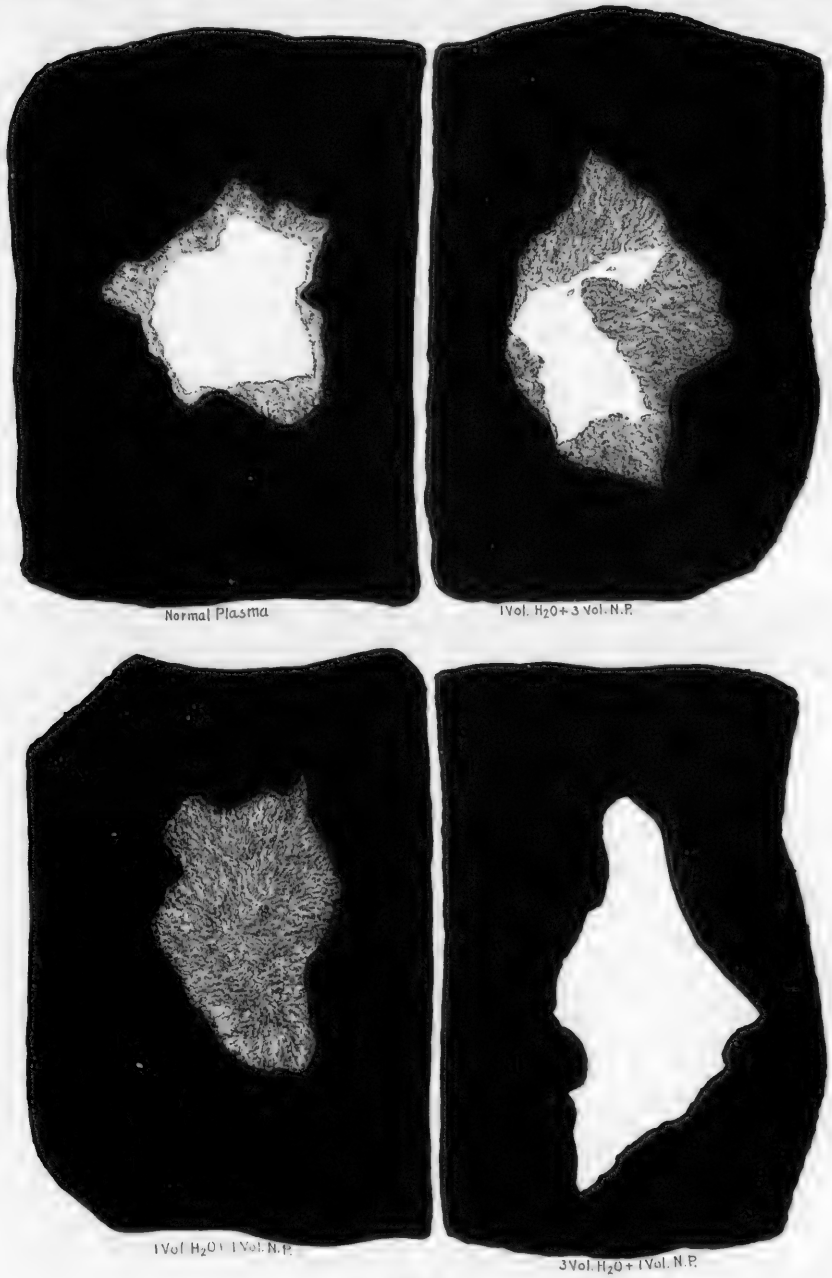

Normal Plasma
1Vol. H_2O+3 Vol. N.P.
1Vol H_2O+1Vol. N.P.
3Vol. H_2O+1Vol. N.P.

FIG. 1.

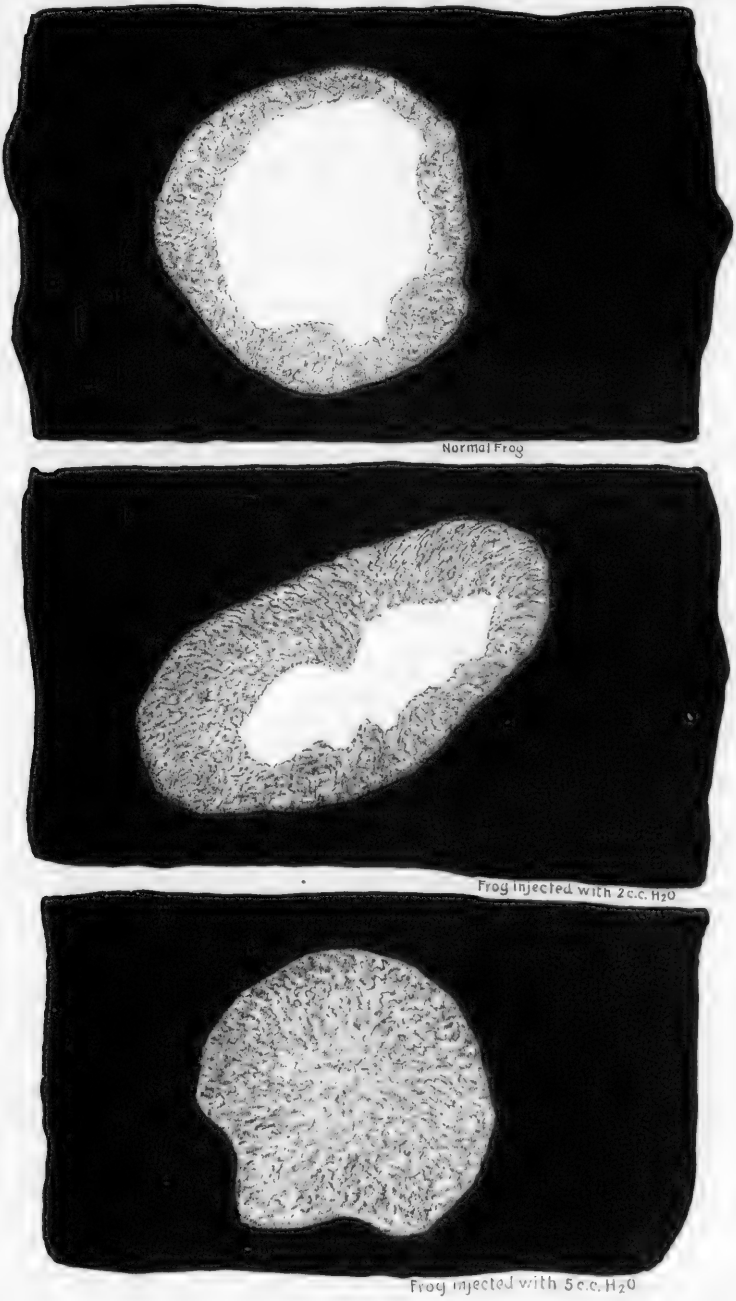

Normal Frog
Frog injected with 2 c.c. H_2O
Frog injected with 5 c.c. H_2O

FIG. 2.

The frogs that received injections of distilled water showed great acceleration of epithelial proliferation, which was maximal in the animals having received from five to ten cubic centimeters of water (figure 2).

These experiments serve to demonstrate the important point that the healing of epidermal wounds of the frog takes place in a practically identical manner in cultures *in vitro* and in the body of the animal. They show further that the rate of growth of new epi-

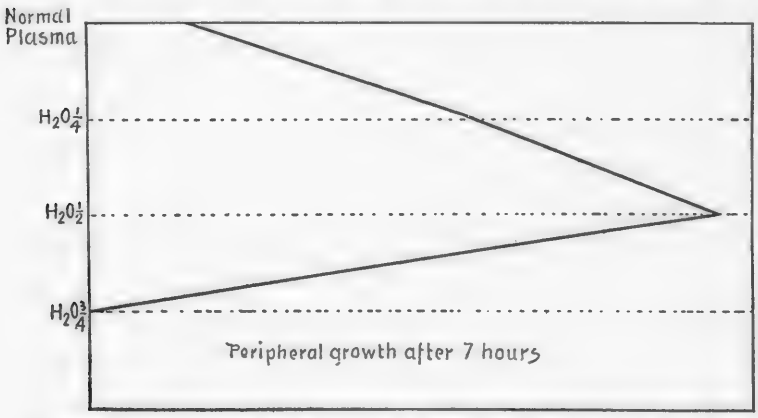


CHART 1. Variations of the peripheral growth of fragments of frog's skin in different plasmas.

thelium which constitutes the process of cicatrization is accelerated by a diluted medium, both *in vitro* and *in vivo*. Thus, by increasing or diminishing the degree of dilution of the medium, or by injecting distilled water or hypertonic solutions into the animal, the rate of growth can, and will up to a certain point, be accelerated or retarded.

EXPLANATION OF PLATES.

PLATE LXXIII.

FIG. 1. Camera lucida drawings showing the differences of epithelial growth in wounds in cutaneous fragments cultivated for eighteen hours in normal and diluted plasmas.

PLATE LXXIV.

FIG. 2. Camera lucida drawings showing the differences, after eighteen hours, of epithelial growth in wounds made in the skin of a normal frog and in two frogs injected with distilled water.

ON THE PHYSICOCHEMICAL REGULATION OF THE GROWTH OF TISSUES.

The Effects of the Dilution of the Medium on the Growth of the Spleen.*

By ALEXIS CARREL and MONTROSE T. BURROWS.

(*From the Laboratories of the Rockefeller Institute for Medical Research, New York.*)

Plates LXXV and LXXVI.

INTRODUCTION.

Since the appearance of the work of Jacques Loeb, it is well known that the rate of growth of certain marine organisms is markedly influenced by the physicochemical conditions of the water. In alkaline sea-water, the fertilized eggs of the sea-urchin develop more quickly than in normal sea-water. When tubularia are deposited in hypotonic sea-water, their growth is considerably increased. The experiments of Loeb have demonstrated the extreme sensitiveness of living cells to variations in concentration of the hydroxyl and hydrogen ions, and the importance of the osmotic tension of the water for the growth of the organisms.

The growth of the tissues of mammals is probably controlled by the conditions of the interstitial lymph in the same way that the growth of the egg of the sea-urchin is influenced by the conditions of the water. The organic and inorganic components of the blood are doubtless the most important of the physicochemical mechanisms which regulate the growth of the body. Therefore it may be assumed that the facts discovered by Loeb in the lower marine organisms are the expressions of general laws which control the development of the tissues and organs of the higher animals as well.

Since the tissues, in their development, must adapt themselves to the morphological plan of the organism, their growth must be con-

*Received for publication, March 17, 1911.

stantly regulated by some unknown factors. This regulation may be caused by certain chemical compounds contained in the blood and the interstitial lymph. Until this year, there was no method for studying the action of the changes in the composition of the blood on the rate of growth. It became possible by the development of the method of cultivation of tissues *in vitro*.

We assumed that the growth of tissues in normal plasma could be compared to the growth of the same tissue in the organism. Then we attempted to determine what physicochemical factors could activate the rate of growth. It was logical to suppose that a medium more efficient than normal plasma could be found, as the tissues in the organism certainly do not meet with the best possible conditions of growth. Thus, if the blood were the best possible medium, the cells would grow without restraint, the organs and tissues would lose their relative size and morphology, and the whole body would become monstrous. Therefore, it may be assumed that the power of growth is kept under constant restraint, that every organ is compelled to follow the morphological plan of the organism, and that normal plasma is far from being the optimum medium for the culture of normal cells. A better medium can probably be obtained easily by modifying in many ways the conditions of the plasma.

We began to study the variations of growth of a few tissues in plasma, the conditions of which had been modified. In this article, we shall describe only the modifications of the rate of the growth of the spleen, brought about by the modification of the dilution of the culture medium, and of its concentration in sodium chlorid.

METHOD.

Our method consisted in cultivating the spleen of adult chickens or of fourteen or sixteen day chick embryos in normal plasma and in plasma of which the osmotic tension had been modified. Although the growth of a culture may be influenced by many causes, nevertheless the results of a given series must be accurately compared. Therefore the details of the technique must be carefully established, as it is of great importance not to consider a merely accidental variation of growth as due to the composition of the plasma.

The rate of growth of a fragment of tissue, cultivated *in vitro*, may be influenced by causes that are inherent to tissue, or to the preparation and preservation of the cultures. The condition of the tissue, its size and thickness, the manner in which it has been cut, the period which elapses between the interruption of the circulation and the imbedding in plasma, the duration of the exposure to air, the degree and duration of the chilling, etc., may have an influence on the rate and extent of growth. The preparation and the preservation of the cultures, and some details of the technique, often affect the growth. The nature of the plasma, the size and thickness of the drop, the dimensions of the hollow slides, the hygrometric condition of the air, the time elapsing between the imbedding of the tissue in plasma and the sealing of the hollow slides, the temperature of the incubator, and the variations of temperature according to the different parts of the incubator, etc., may cause important variations. Unless great care is taken to eliminate, as far as possible, these sources of error, the results of the cultures in different media can not be accurately compared. It was, therefore, necessary to give greater precision to the technique, in order that the growth of the cultures in a given medium should be rendered uniform.

Preparation of the Plasma.—A large quantity of blood was taken from a chicken in order that several series of experiments could be made with the same plasma. The plasma was prepared by the ordinary method, and part of it was used for the control cultures. The other part was rendered hypotonic or hypertonic by the addition of distilled water or sodium chlorid. The hypotonic plasma was composed of normal plasma diluted with one fifth, two fifths, one half, three fifths, and four fifths distilled water. The hypertonic plasma was obtained by adding four volumes of normal plasma to one volume of a solution of 0.015, 0.02, 0.03, and 0.04 of sodium chlorid in distilled water. If we suppose that normal chicken plasma contained 0.008 sodium chlorid, the hypertonic plasma contained 0.0094, 0.0104, 0.0124, and 0.0144 sodium chlorid, respectively.

Preparation of the Spleen.—The spleen was taken either from an adult chicken, or, as was more generally the case, from a fourteen or sixteen day old chick embryo. Great care was taken to dissect

the tissue very rapidly as soon as the circulation was interrupted, or the egg opened, in order to diminish, as much as possible, the period of exposure of the tissue. A small fragment of spleen was divided into eight or ten smaller pieces of equal size. The pieces were rapidly deposited on the cover glass, and covered with the hypertonic or hypotonic plasma. One control culture in normal plasma was made at the beginning and at the end of each group. The conditions and the preparation of the tissues of the same group of cultures were almost identical, and the results could therefore be legitimately compared.

After the fragments of tissues had been covered with plasma, the cover glasses were quickly placed on hollow slides and sealed. If some of the cover glasses remained exposed to the air for a longer time than the others, the plasma evaporated and became more concentrated, and the results were modified. The amount of plasma and the dimensions of the confined atmosphere of the hollow slides must be the same for each culture of a group.

The cultures, divided into several groups of eight or ten slides, were deposited in the incubator. The location of the slides near the wall or near the door of the incubator may have an influence on the rate of growth. It is necessary that the temperature should remain exactly the same for each slide of a group.

The results were examined a few hours after the preparation of the culture. Cells of the fetal spleen started to migrate immediately, without any latent period, and after two hours a large area of densely packed cells could be seen around the original tissue. Often it covered all the culture medium in twenty-four or thirty-six hours. The rate of growth was appreciated by the changes of the dimensions of the ring of new tissue surrounding the original fragments. It is important to cut the tissue into as regular fragments as possible, in order to obtain an equal growth all around it. The peripheral part of the new tissue appeared then as a regular circumference, and it was easy to calculate the area covered by the cells which had wandered out from the tissue or which multiplied in the culture medium. It was, then, possible to know the rate of growth in the different media. Often several series of camera lucida drawings were made of the growing specimens.

The main source of error was changes in growth produced by an accident of technique. When the control cultures showed widely different conditions of growth, or when some of the fragments of tissues did not grow at all, the technique was considered defective, the variations of growth were interpreted as due to an accident, and the group which presented the irregularities was discarded. Although the different groups of a series can sometimes be compared, it is preferable to compare only the different cultures of the same group and their controls. Even by using these precautions, all sources of error are not eliminated, but they are greatly reduced. They can be suppressed only by comparing many experiments and discarding the exceptional results. As the technique is complicated, there is always the possibility of an accident. Conclusions must not be based on one group of experiments only; they must be controlled by the results of another group, for the cultures of tissues *in vitro* give comparable results only when the greatest care is taken to eliminate all technical errors.

EXPERIMENTS AND RESULTS.

Small fragments of the spleens of adult chickens and chick embryos were cultivated in normal, hypertonic, and hypotonic plasma.

1. *Cultures in Normal Plasma.*—The fetal spleen grew without a latent period. After two hours, the tissue was already surrounded by a thick crown of cells. The growth of adult spleen was very much slower and became apparent ordinarily after twelve hours. The cultures in normal plasma were used as controls for the cultures in hypertonic and hypotonic plasma.

2. *Cultures in Hypertonic Plasma.*—The spleen was very sensitive to the action of hypertonic plasma. The fetal spleen still grew in plasma containing 0.0094 sodium chlorid, but the growth was much less extensive than in normal plasma. In one case it was sixteen times smaller. In plasma containing 0.0104 sodium chlorid, it grew slightly, and not at all in the more hypertonic plasma. The adult spleen showed slightly different variations (figure 1, *a, b, c, d, e*). For instance, in series S_5 the growth in normal plasma was three hundred and fifty and three hundred and thirty, while the growth was three hundred and ninety-two in plasma containing

0.0094 sodium chlorid, sixty-three in plasma containing 0.0104 sodium chlorid, and nothing at all in the still more hypertonic plasma. The results observed in the other groups of cultures showed also that hypertonic plasma diminishes greatly the growth of the spleen. Nevertheless, some activation of the growth may be observed in cultures in slightly hypertonic plasma.

Similar results have been obtained in the cultivation of other tissues in hypertonic plasma. The growth was also retarded. The

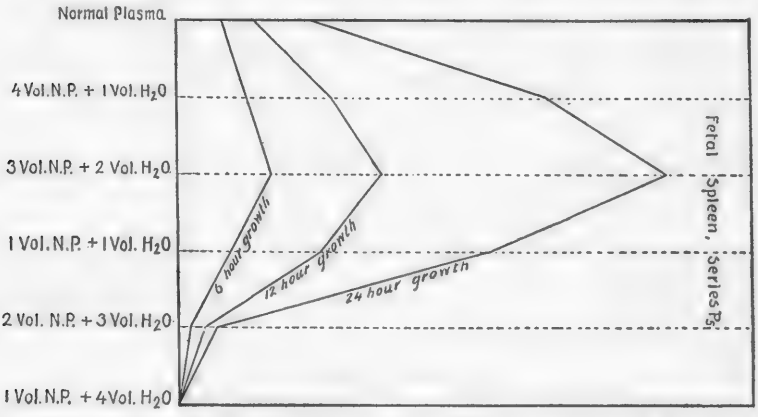

Normal Plasma
4 Vol. N.P. + 1 Vol. H_2O
3 Vol. N.P. + 2 Vol. H_2O
1 Vol. N.P. + 1 Vol. H_2O
2 Vol. N.P. + 3 Vol. H_2O
1 Vol. N.P. + 4 Vol. H_2O
6 hour growth
12 hour growth
24 hour growth
Fetal Spleen, Series P5

CHART I shows the variations in growth, at different periods after the preparation of the culture, of fragments of fetal spleen cultivated in normal and in diluted plasma.

skin reacted in a different manner and was considerably activated in slightly hypertonic plasma. It is probable that the sensitiveness of tissues to a hypertonic medium varies according to their nature.

3. *Cultures in Hypotonic Plasma.*—A slight dilution of the plasma always produced acceleration of growth. The growth of the spleen in plasma containing one fifth distilled water was very much greater than in normal plasma (figure 2, *a* and *b*). It was still greater in plasma containing two fifths distilled water (figure 2, *c*), and when the plasma was diluted still more, the area of growth diminished progressively; but in plasma containing one half distilled water, it was still larger than in normal plasma (figure 2, *d*). In plasma containing three fifths distilled water, fetal spleen

could still grow, although very much less than in normal plasma (figure 2, *e*). Adult spleen did not grow at all in the plasma containing three fifths and four fifths distilled water.

In the drawings (figures 1 and 2), in the chart, and in the table are summarized the action of hypotonic and hypertonic plasma on fetal and adult spleen.

Tissue.	Time of cultivation.	Normal plasma.	+ H_2O $\frac{1}{5}$	+ H_2O $\frac{2}{5}$	+ H_2O $\frac{1}{2}$	+ H_2O $\frac{3}{5}$	+ H_2O $\frac{4}{5}$
Fetal spleen	6 hours	1130	1760	2488	1318	329	Few cells
Series P_5	12 hours	2010	4005	5356	3732	716	Few cells
	24 hours	3540	9676	12741	7050	990	Few cells
Fetal spleen	4 hours	120 $\times\pi$	229 $\times\pi$	483 $\times\pi$	440 $\times\pi$	350 $\times\pi$	0
Series S_5	9 hours	960 $\times\pi$	2600 $\times\pi$	3430 $\times\pi$	2208 $\times\pi$	2115 $\times\pi$	0
Adult spleen							
Series S_5	24 hours	255 $\times\pi$	960 $\times\pi$	1520 $\times\pi$	168 $\times\pi$	0	0

Analogous results were observed when the skin, heart, and liver of chickens were cultivated in hypotonic plasma. In the experiments made in this laboratory by Dr. Ruth on the cicatrization *in vitro* of cutaneous wounds of adult frogs, it was also found that plasma containing one half distilled water accelerated very much the epithelial growth.

CONCLUSIONS.

It may be concluded that the degree of dilution of the culture medium has a marked influence on the rate of growth of splenic tissue. The maximum acceleration was obtained in a medium composed of three volumes of normal plasma and two volumes of distilled water. The growth in this hypotonic plasma was very much larger than in normal plasma. On the contrary, the growth of the spleen in hypertonic plasma was always less than in normal plasma.

In other experiments, we found that in diluted plasma there was also an acceleration of the growth of the skin, the heart, and the liver of chickens. The skin of adult frogs also grew more actively in this plasma.

The optimum degree of dilution varied according to the nature of the tissues and to the species of the animals. While the plasma containing two fifths distilled water produced the largest growth of splenic tissue, a slightly less diluted medium was more favorable for the liver and the heart, and generally for the skin also. The

action of hypertonic plasma varied also in a large measure. While the spleen did not grow at all in the medium containing 0.0124 and 0.0144 sodium chlorid, the skin, on the other hand, could stand a high concentration of the sodium chlorid. Even its growth was activated in media containing 0.0094 and 0.0124 sodium chlorid and was greater than with normal plasma. The spleen of kittens was very easily affected by the changes of the dilution of the plasma, while the skin of the frog presented its best growth in plasma containing one half distilled water. Marked variations in the sensitiveness of tissues to hypertonic and hypotonic media will probably be observed in animals of different species.

From these experiments, three conclusions can be drawn: namely, that certain laws of growth, discovered by Loeb, in lower organisms are true also for higher organisms; that normal plasma is not the optimum medium for the growth of tissue; and that each tissue has probably its optimum medium.

The growth of the spleen is, without doubt, considerably modified by the variations of the dilution and perhaps of the osmotic tension of the plasma. It is possible then that the influence of osmotic tension, discovered by Loeb, in the growth of certain organisms, is a general law applicable as well to higher forms of life—frogs, cats, and chickens—as to lower organisms—tubularia and sea-urchins. In placing tubularia in different dilutions of sea-water and distilled water, Loeb found that the greatest rate of regeneration was observed when two volumes of distilled water were added to three volumes of sea-water. But fertilized eggs of sea-urchins were more sensitive to the action of hypertonic plasma, and they all died in a dilution of sea-water with two fifths distilled water. If only one fifth distilled water was added to the sea-water they developed normally. We found that the cells of certain tissues of the chicken follow a similar rule, since the maximal growth of the spleen is obtained in plasma containing two fifths distilled water, while other tissues grow better in a less hypotonic medium.

Normal plasma is certainly not the ideal medium for the growth of tissues, since slight modifications of the tension, the alkalinity, or the addition of certain inorganic salts to normal plasma, increase the rate of the growth of tissues.

It is possible, also, that the composition of an optimum medium would be different for each kind of tissue, and that no tissue meets inside of the organism with the best possible conditions for its development. If a tissue or an organ found in the body the best possible medium, it would grow indefinitely, reach an enormous size, and become a source of danger to the organism itself. Nevertheless, it would be very important to determine the composition of the medium that each organ and each tissue requires for its maximal development. Thus favorable conditions could possibly be given to a tissue temporarily, without interfering greatly with the nutrition of the other tissues of the organism. For instance, the peripheral part of a cut nerve often does not regenerate because a fibrous scar prevents the outgrowth of the axis cylinders from the central end. If the conditions of the interstitial lymph or of the culture medium which activate the growth of the nervous cells were known, we might accelerate artificially the rate of growth of the axis cylinders, and cause them to penetrate the peripheral end of the nerve before the formation of a scar, and thus promote regeneration.

It would, therefore, be of great value to determine for each tissue the medium which permits its maximal growth. Even if the accomplishment of this does not lead to any immediate practical application, the knowledge of the optimum conditions may lead to the discovery of some of the physicochemical mechanisms which regulate the development of the organs and compel them to comply with the morphological plan of the organism.

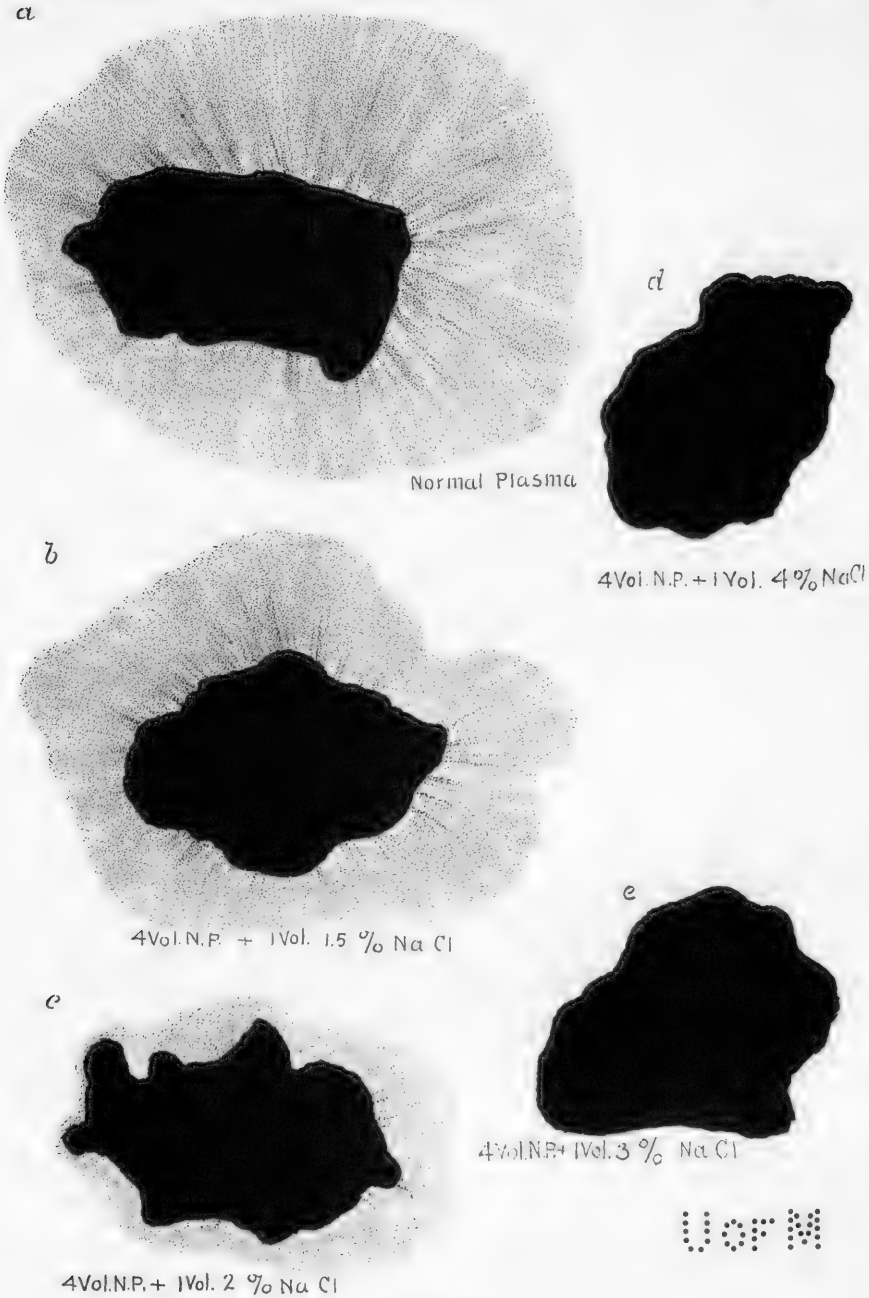
EXPLANATION OF PLATES.

PLATE LXXV.

FIG. 1. Camera lucida drawings showing the variations in growth, twenty-four hours after the preparation of the cultures, of fragments (*a, b, c, d, e*) of adult spleen cultivated in normal plasma and in plasma of which the concentration in sodium chlorid was increased.

PLATE LXXVI.

FIG. 2. Camera lucida drawings showing the variations in growth, twelve hours after the preparation of the cultures, of fragments (*a, b, c, d, e, f*) of fetal spleen cultivated in normal and in diluted plasma.


a
Normal Plasma
d
4Vol. N.P. + 1Vol. 4% NaCl
b
4Vol. N.P. + 1Vol. 1.5 % Na Cl
e
4Vol. N.P. + 1Vol. 3 % Na Cl
c
4Vol. N.P. + 1Vol. 2 % Na Cl

U of M

FIG. 1.

Mau

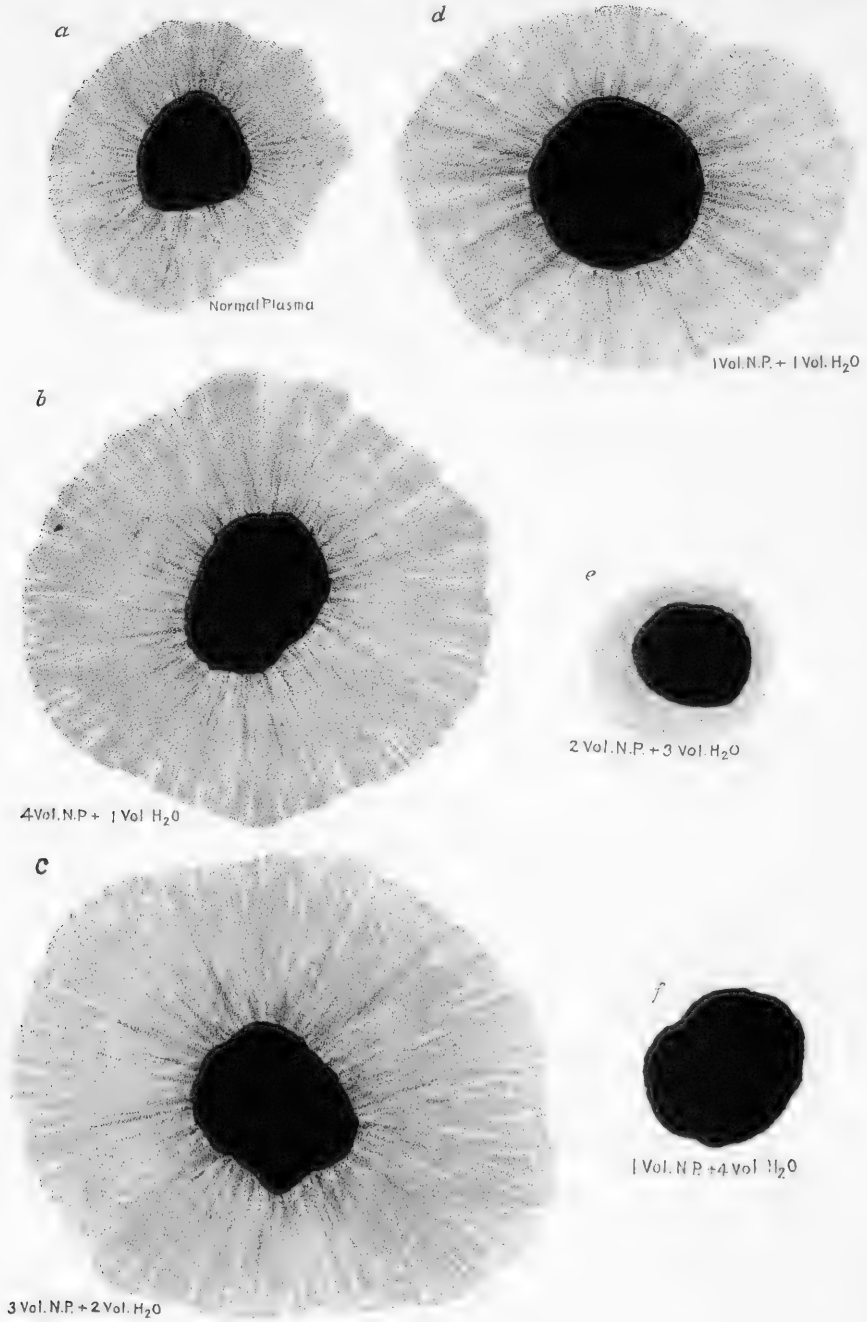

a
Normal Plasma
d
1 Vol. N.P. + 1 Vol. H_2O
b
4 Vol. N.P. + 1 Vol H_2O
e
2 Vol. N.P. + 3 Vol. H_2O
c
3 Vol. N.P. + 2 Vol. H_2O
f
1 Vol. N.P. + 4 Vol H_2O

FIG. 2.

CULTIVATION IN VITRO OF MALIGNANT TUMORS.*

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PLATE LXXVII.

The growth of malignant tumors in cultures outside the body has been studied in a large number of experiments. The technique employed has already been described[1] and need not be repeated here. We propose, in this article, to describe the growth characteristics of the different varieties of tumors employed for cultivation.

The tumors employed were as follows: the Rous chicken sarcoma (2), the Ehrlich and the Jensen rat sarcomata, the Flexner-Jobling rat carcinoma, a dog carcinoma, and human sarcomata (3) and carcinomata. By far the largest number of tests was made with the Rous chicken sarcoma, of which nearly four hundred cultures were studied. The next largest number of tests was made in connection with the malignant human tumors, of which nearly two hundred cultures were made. Many cultures were made of the other varieties.

CULTIVATION OF CHICKEN SARCOMA.

The Rous chicken sarcoma grows rapidly in cultures. Moreover, its cells are actively migratory and quickly begin to wander from the tissue fragment into the plasmatic medium. Within two hours the emigration has begun, and within the next few hours proliferation has advanced. The rate of proliferation is shown by the fact that in culture 4 of series M_2, the area covered by the growth in twenty-four hours was forty times that of the original fragment. It is common for the growth area to reach fifteen to thirty times that of the original fragment within twenty-four hours.[2]

Tumor tissue does not necessarily exceed all other tissues in

* Received for publication, March 18, 1911.

[1] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 387.

[2] Carrel and Burrows, *Jour. Am. Med. Assn.*, 1910, lv, 1554.

rapidity of growth in cultures. Indeed, we have found that embryonic tissue, especially splenic, exceeds in this respect the chicken sarcoma.

The factors governing the rate of growth are numerous, as we have already pointed out.[3] In the case of the Rous sarcoma, the state of preservation of the cells is highly important. Many failures could be traced to degenerations of the tumors, particularly myxomatous changes, affecting the fragments cultivated. But the nature of the plasma also affects the result: the most rapid growth was secured with autogenic plasma, and less rapid proliferation with homogenic plasma. Plasma from another chicken bearing sarcoma was less adapted, and sometimes was entirely unsuited to growth; while the addition of minute quantities of sarcomatous extract to normal plasma produced acceleration of growth.[4] Hence, the conditions are complex and will require much patient and ingenious observation for their elucidation.

The appearance of the growing tissue is striking. About the original fragment a continuous and dense layer of elongated and round cells collect, which quickly invade a large part of the medium. The new growth is divisible into two parts. The inner part is composed of radiating, spindle and round cells, the outer part almost wholly of ameboid round cells. The outer layer of cells is surrounded by red corpuscles and debris, doubtless carried mechanically before the advancing cells. The disposition of the new cells may be in an horizontal plane or in the form of a concavity with the greatest depth at the edge of the tissue fragment.

The life of the cultures is short. By the expiration of forty-eight hours, the rate of growth is generally much slower and granules appear in the cells, after which cellular disintegration sets in. A further difference occurs: in cultures showing slow growth, the spindle cells predominate, and in those showing rapid growth, the round cells predominate.

The round cells contain a clear cytoplasm enclosing a few refractile granules and a nucleus often difficult to see in fresh preparations; their ameboid activity is lively. The spindle cells possess

[3] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 416.
[4] Carrel and Burrows, *Jour. Am. Med. Assn.*, 1911, lvi, 32.

clear cytoplasm, enclosing many refractile granules (figure 1) which may cover and obscure the nucleus. They stain red with Sudan III and black with osmic acid, and hence are of the general nature of fatty or lipoidal substances. Similar granules arise in the normal tissue cells of the chicken during cultivation. The ameboid cells are actively phagocytic. Culture 12, series F, consisted of sarcomatous and pigmented cells derived from a fetal eye of a chicken. The sarcoma cells wandered in and out among the pigmented cells, and gradually took up the dark pigment, so that after forty-eight hours they were stuffed with it.

The increase in cells is associated with karyokinetic division of the nuclei. During the first twenty-four hours, this nuclear division is observed chiefly in the inner area, close to the original fragment; but later it occurs throughout the growing area. At about the period of cessation of growth and of life of the culture, it occurs only at the outer edge. This demonstration constitutes the final proof that in the cultivation of tissues outside the body actual multiplication of cells and growth of tissue occur.

Finally, it may be stated that we found no characteristic for sarcoma cells, as such, that was not also to be found in some normal tissue cells of the chick.

CULTIVATION OF RAT SARCOMATA.

Sarcomata of the rat, so far as they are represented by Jensen's and Ehrlich's strains, behave in cultures very much as the Rous chicken sarcoma does. There are, however, some important differences of detail. The growth begins usually after a latent period of from four to twelve hours, and continues from four to six days. The new cells are round and spindle-shaped, and the fragments become surrounded by a dense network of real tissue. Thus it comes about that the line of demarcation between the original fragment and the new growth is almost invisible. In process of growth the round cells occupy the most advanced position in the medium, and are followed by the spindle cells. The morphology of the tumor cells is distinct from that of the connective tissue cells. Karyokinesis has been present in specimens which have grown for as great a time as six to eight days.

CULTIVATION OF HUMAN SARCOMATA.

Three human sarcomata were subjected to cultivation. They consisted of two giant-celled tumors and a fibrosarcoma. We failed to cultivate the giant-celled tumors, probably for the reason that they caused rapid liquefaction of the medium. The medium about the fibrosarcoma underwent partial liquefaction, but as a thin and firm clot adhered to the cover-glass, growth was still possible, and after a latent period varying from six to twenty-four hours, it took place. Polygonal and spindle-shaped cells wandered into the medium and remained isolated and did not produce a dense new tissue, as did the sarcoma of the rat.[5]

On account of the extreme tenuity of the plasmatic medium it was possible to follow all the details connected with the proliferating cells. The fusiform cells developed sharp processes, and their clear protoplasm was seen to become filled with refractile granules that surrounded the large central nuclei. The cytoplasm also came to contain small round or irregular bodies, of which some showed side knobs like buds (figure 2), the nature of which is still undetermined. The bodies were opaque and occupied vacuoles in the living cells; in stained preparations, they took on a pale tone from the nuclear dye. Within these pale bodies, a far darker and minute spot sometimes appeared. Similar cell inclusions were observed in the cells of the original, uncultivated tumor.

CULTIVATION OF CARCINOMATA.

Cultivation of carcinoma was attempted with (1) an adenocarcinoma of the breast of an old dog (bitch), (2) the Flexner-Jobling rat carcinoma, and (3) several carcinomata of human beings; one from the lip, and the others from the male and female breasts.

Success was achieved with the dog tumor, in which growth of the adenomatous portion was noted to begin on the third day. The growth consisted of blunt tubules or columns of cells presenting an epithelial-like appearance. The tubules resembled those which we have observed in cultures of the thyroid gland[6] and the kidney. The cells composing them were round or polygonal; the nuclei were

[5] Carrel and Burrows, *Jour. Am. Med. Assn.*, 1910, lv, 1732.
[6] Carrel and Burrows, *Jour. Exper. Med.*, 1911, xiii, 416.

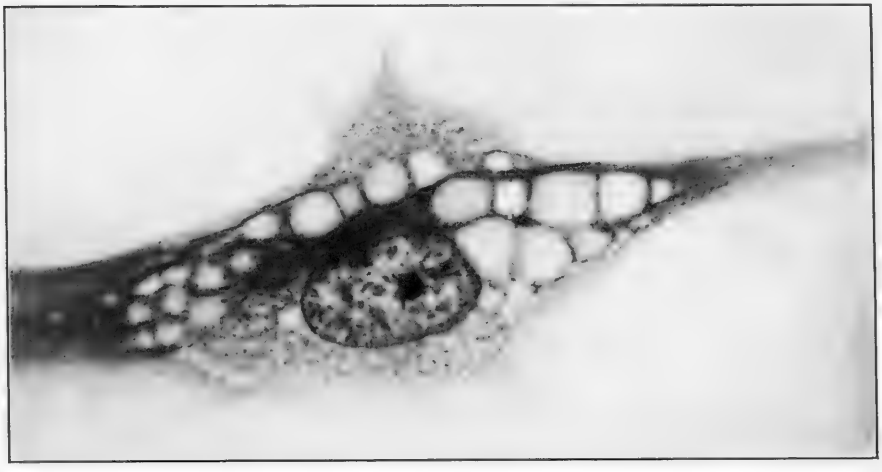

FIG. 1.

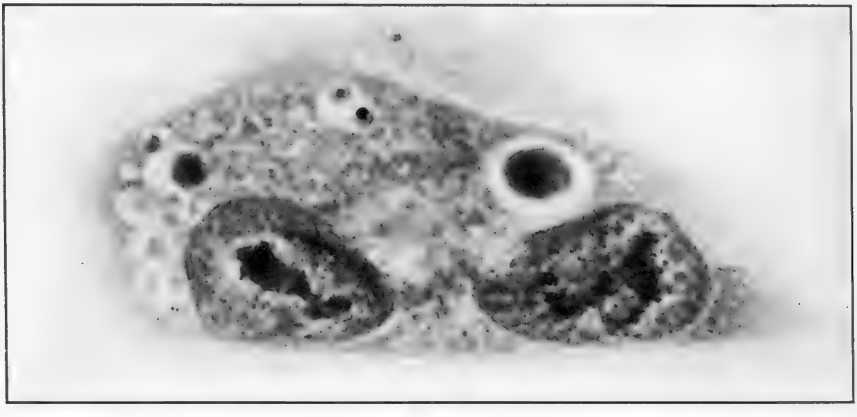

FIG. 2.

central; the cytoplasm was clear and homogeneous, except for small refractile granules. We have observed cells similar to these in cultures of the skin and liver.

In the Flexner-Jobling rat carcinoma the growth was composed of epithelial-like cells resembling those observed in the cultures of the spontaneous carcinoma of the dog.

Lambert and Hanes have succeeded in cultivating a mouse carcinoma.[7]

Thus far we have not had signal success with the cultivation of human carcinoma, for the reason that the six specimens with which we worked rapidly liquefied the plasmatic medium. Some growth was obtained from a small carcinoma of the breast; generally liquefaction occurred both in the cultures prepared from the tumor and from the pectoral muscle and lymphatic glands. In order to succeed with cultures of human carcinoma it will be necessary to master and overcome the process of liquefaction. We have already found that not all human tissues cause liquefaction of the plasmatic medium, so we believe that the conditions can be discovered and prevented.

It can therefore be concluded that experimental malignant tumors grow *in vitro* extensively, and that the cultivation of human tumors is also possible, although much more difficult on account of the liquefaction of the plasma.

EXPLANATION OF PLATE LXXVII.

FIG. 1. An isolated spindle cell from a culture of the Rous chicken sarcoma. The large clear spaces mark the sites of dissolved fat granules. Stained with hematoxylin and eosin.

FIG. 2. An isolated multinuclear cell with inclusions from a culture of human fibrosarcoma of the fibula. Stained with hematoxylin and eosin.

[7] Lambert and Hanes, *Proc. Soc. Exper. Biol. and Med.*, 1911, viii, 59.

THE BEHAVIOR OF BACILLUS LEPRÆ IN COLD-BLOODED ANIMALS.*

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PLATE LXXVIII.

At the time the study of experimental leprosy in various species of mammals was begun by Dr. Duval, many species of cold-blooded animals were inoculated with the same strain of *Bacillus lepræ* that was used for the mammals. The culture was obtained in pure growth twelve months previously by Dr. Duval, who has described its characters in detail.[1] In order to afford a basis of comparison, a drawing of the normal organism as it appears in culture is appended (figure 1).

In the experiments presented here, seven tadpoles, nine frogs, four turtles, four snakes, twenty-six gold fish, and thirty-two salt water fish of various kinds were employed, either for inoculation or for feeding with cultures of the bacillus.

All the fish used were very active and apparently healthy when inoculated. With the exception of those employed in the feeding experiments, all were inoculated subcutaneously on the right side of the back, midway between the tail and the head. The culture emulsion was injected immediately below the skin, penetration into the muscle being avoided as much as possible. The amounts of the bacterial suspension used varied according to the size of the animal. In many instances, a slight swelling along the point of inoculation followed the injection, otherwise the animals showed nothing of note. From four to six days following the inoculation, there was sometimes seen an ecchymotic area at the site of puncture, which remained for a variable length of time, but had usually disappeared

*Read before the American Association of Pathologists and Bacteriologists, Chicago, April 14, 1911. Received for publication March 17, 1911.

[1] *Jour. Exper. Med.*, 1910, xii, 649.

by the tenth day. In no instance were there further macroscopic changes, either in the form of tumefaction, discoloration, etc., or in a lessened activity of the animals.

The tadpoles, frogs, and snakes were inoculated into the peritoneal cavity or subcutaneously. In these animals no external evidences of the inoculation could be seen at any time.

Two turtles were inoculated below the skin of the neck, and two intraperitoneally; one turtle injected intraperitoneally died two days later.

In the feeding experiments, the same strain of *Bacillus lepræ* was employed.

EXPERIMENTAL OBSERVATIONS.

PROPAGATION BY DIRECT INOCULATION FROM CULTURES.

Experiment I.—Six gold fish (*Carassius auratus*) 10 cm. long were inoculated subcutaneously with three minims each of a suspension of *B. lepræ* in normal saline solution. There was no perceptible effect from the inoculation. Fish A was killed seventeen days after inoculation; B, twenty days; C, twenty-three days; D, thirty days; E, thirty-nine days; and F, fifty days after inoculation.

There was nothing upon the skin or about the scales of the animals, when killed, to denote the existence of underlying lesions. The skin was found adherent along the track of inoculation and on each side extending to the spinous processes of the vertebræ above and to the abdomen below. Upon breaking the adhesions, bits of tissue were carried with it. The epidermis on the left side was not adherent. The ragged surface left after removing the skin was swollen and slighty darker in color than the corresponding area on the other side, and was covered with a gelatinous substance, which was greater in fish killed after twenty days than in those killed later. The appearance suggested a fibrinous exudate; but on microscopic examination no fibrin or other evidence of acute inflammation existed. The films showed, however, masses of bacilli and many large and smaller cells with a single reticulated nucleus, within the cytoplasm of which were large numbers of acid-fast organisms. The smaller cells, measuring about twenty microns contained few or no bacilli, while those measuring from 100 to 130 microns contained as many as one to two hundred. Films from fish killed early showed fewer cells containing bacilli than those killed after longer periods. In the muscle tissue on the injected side were several minute grayish irregular areas. These areas were round or oval, or they appeared as streaks radiating from the exudate below the skin. They were more distinct in the fish killed on the seventeenth, twentieth, and twenty-third days, and could hardly be seen in those killed thirty-nine and fifty days after inoculation. On the side opposite the inoculation, no lesions could be seen, although films showed cells similar to those found on the inoculated side. These cells, suggesting lepra cells, were smaller and less numerous, and contained fewer bacilli than those about the site of inoculation. The numbers of acid-fast bacilli were taken to indicate

multiplication. Multiplication was much more striking in the fish killed after the longer periods.

The bacilli recovered from the fish agreed in morphology with the organism inoculated. They remained short, unbeaded, and coccoid, even in animals killed fifty days after the inoculation (figure 2).

Experiment II.—Five spots (*Leiostomus xanthurus*), two spotted sea-trout (*Cynoscion nebulosus*), twenty croakers (*Micropogon undulatus*), and five striped mullets (*Mugil cephalus*), were inoculated below the skin of the side of the back with three minims of the same suspension of *B. lepræ*. There were no immediate effects. One spot, one trout, and three croakers died four days later. In all five, there was a marked diminution in the number of bacilli injected. In two animals, the spot and one croaker, the organisms were found with difficulty, but they retained their original morphology. Some were elongated with pointed ends but unbeaded, while a few showed polar stained granules or massed chromatin. Lepra cells were absent. The twenty-seven surviving fish were killed on the seventh, fourteenth, and eighteenth days after inoculation.

One trout, one spot, and four croakers killed on the seventh day showed no external evidence of infection until examined by transmitted light, when distinct opaque areas could be seen below the skin extending radially from the site of inoculation, which were more marked in the croakers than in the trout, and barely visible in the spot. Upon opening the fish, a thick gelatinous exudate was found below the skin extending for some distance along the sheath of the muscles. Some of the opaque areas seen by transmitted light were quite large (3 mm.), and some (about 1 mm.) appeared as small as pin-head sized areas in the outer border of the muscle of the bull croaker. The trout lesions were fairly well marked and appeared as opaque bands following the muscle sheaths and radiating from the gelatinous material below the skin.

While lepra cells were absent from fish dying four days after inoculation, they were numerous in the animals killed after longer periods.

The smears made from the jelly-like material showed innumerable smaller and many larger cells. The nuclei were reticulated, oblong, and pushed to the edge of the cell by the bacilli. They retained normal staining properties and showed no evidences of degeneration. The cells were similar to those found in the gold fish; the larger ones contained masses of acid-fast bacilli, while the smaller ones contained only a few or none.

While a few single bacilli were present in the stained preparations, the greatest numbers occurred as colonies within the lepra cells. It was not uncommon to find two or more large and smaller masses of leprosy bacilli in a single microscopic field. Extracellular bacterial masses, evidently mechanically dislodged from the cells, also occurred. A few of the bacilli were short and unbeaded, but the great majority were long, thick, and beaded. Some showed two, and others as many as five, distinct chromatin beads. Some of the clear spaces between the red staining granules were distinctly "bellied," suggesting spores (figure 8). A noteworthy change in the organisms relates to their thickening, some bacilli being fully one third thicker than usual. Acid-fast bacilli were found scattered throughout the whole body, but less in the spots than in the other fish. In the fish killed fourteen days after injection, the lesions, though

plainly to be seen by transmitted light, were more diffuse, and hence less opaque. Still they were similar in every way to those present in the fish killed a week earlier. The number of larger lepra cells had now greatly increased, although many smaller ones still occurred. That multiplication had gone on is shown by the larger size and greater number of the globi. The bacilli appeared the same as in the fish killed a week earlier, with the possible exception that fewer retained the coccoid form.

The remaining fish (five mullets, one spot, and eight croakers) were killed eighteen days after inoculation. At that time all were active and showed no external indication of infection.

The five mullets showed no acid-fast bacilli. Earlier examinations of this species of fish were not made, and it is therefore difficult to state definitely the time at which the organisms disappeared.

The spot and croakers were without external lesions. On section, the usual tissue changes were present, though apparently less developed than in the fish previously described. The spot showed very small areas quite deep in the muscle. But the inoculated side in the spot and in the ordinary croaker was darker and slightly larger than the opposite one.

Lepra cells and bacterial masses and single bacilli were numerous, and the former were large. The scattered bacilli were more numerous than in the seven and fourteen day fish, and the tissues of the opposite side of the spine of these croakers, contained scattered acid-fast organisms. In the bull croaker, infection seemed to be limited to the inoculated area. The morphology of the organisms remained unchanged (figure 8).

Experiment III.—Nine leopard frogs (*Rana pipiens*) were inoculated as follows with three minims of the suspension: four below the skin of the back, three below the skin of the hind leg, and two into the peritoneal cavity. The two frogs injected intraperitoneally died in twenty-four and forty-eight hours respectively.

After five days, two frogs were killed. They had been injected into the back and the hind leg respectively. In both a few leprosy bacilli were found near the point of inoculation. All lesions were absent and the few bacilli present had, in this short time, undergone a complete metamorphosis; the short unbeaded forms had become long, broad, and distinctly beaded, many containing four chromatin masses each.

Two frogs inoculated in the back were killed fifteen days later. They gave no external evidences of infection; but a gelatinous exudate existed, which was, however, not abundant. Smears from the exudate showed many masses of intra- and extracellular acid-fast bacilli. The cells were small, and very few contained more than twenty to thirty, and many less than ten bacilli. Judging from the number of bacilli present it was evident that the organisms had multiplied. The organisms were still fairly long and thick and some showed polar stained granules. In a few bacilli there were three to four granules, although the greatest number had two.

One frog injected subcutaneously into the leg, and two injected into the back were killed thirty days later. No external lesions existed. Upon removing the skin, a thick, steel gray exudate was seen, and the underlying muscle tissue was moderately congested. Smears made from the exudate showed numerous very

large bacterial masses and many large cells containing masses of bacilli. Several thousand organisms were found in almost every field. The great number of organisms compared to that found in animals killed at shorter intervals indicates that they have multiplied profusely. The morphology of the bacilli had almost completely changed to the short unbeaded forms (figure 4). In both of these frogs, the bacilli and a few lepra cells were found in the omentum, and bacilli had invaded still other distant parts.

Experiment IV.—Seven tadpoles (*larvæ, Rana pipiens*) received 0.2 c.c. each of the same culture used in the previous experiments. Four were injected beneath the skin of the back, and three into the peritoneal cavity. All survived the inoculations. Three days later two were killed. The one injected in the back showed locally many acid-fast bacilli, but no exudate or other lesion. In the other tadpole injected intraperitoneally, there was a slight amount of gelatinous exudate covering the viscera which contained many acid-fast bacilli, but no lepra cells with bacillary inclusions. The bacilli in both animals were confined to the inoculated site, and appeared normal. The five remaining tadpoles were killed twenty-eight days later. Two had in this time developed into frogs. In the exudate were large and small masses of bacilli, the former predominating. The masses were contained chiefly inside of lepra cells; there were a few extracellular colonies. Some of the latter consisted of several thousand bacilli, nearly all of which were of the short unbeaded variety. The longest ones were about two thirds as long and slightly thicker than those found in human lesions. Some bacilli contained as many as three or four chromatin masses (figure 6). It was evident, on comparing these animals with those killed previously, that the bacilli had multiplied.

Experiment V.—Four turtles, one being a box-turtle (*Cistudo triunguis*) 10 cm. long, the others geographic terrapins (*Malacoclemmys geographica*) 15 cm. long, were inoculated. The box-turtle and two terrapins were injected intraperitoneally, and the other terrapin subcutaneously. One of the terrapins died two days later. The peritoneal cavity contained the injected organisms of normal appearance.

A terrapin inoculated beneath the skin of the neck was killed on the twentieth day. A viscid exudate was found extending along the entire length of the neck, but no other lesions. However, films made from the peritoneum and the subcutaneous tissues of the hind legs showed a few acid-fast bacilli. These were short, unbeaded, and coccoid. The viscid exudate found in the subcutaneous tissues of the neck consisted chiefly of mononucleated cells, many of which contained acid-fast bacilli, besides which many large bacterial masses occurred.

The box-turtle and the two remaining terrapins were killed thirty-five days after inoculation. The injection site showed a viscid exudate, and in it were found large cells with bacterial inclusions, besides enormous numbers of extracellular bacterial masses and scattered bacilli. The bacilli retained their ordinary appearance (figure 5).

Experiment VI.—Four king snakes (*Ophibolus getulus sayi*) 50 to 75 cm. long were inoculated, two into the peritoneal cavity and two into the subcutaneous tissue. Twelve days later two of these were examined. Neither showed external evidence of infection. The skin of the animal injected subcutaneously was removed, but no lesion could be found about the point of inoculation. Films

made from the subcutaneous tissue and muscle showed a large number of scattered acid-fast bacilli, and a moderate number of lepra cells containing leprosy bacilli. The cells were chiefly of the small type and many showed a few acid-fast bacilli. A few of the single bacilli, and most of the intracellular ones, were short and unbeaded; while most of the extracellular ones varied in size and morphology from the fairly long bipolar variety with pointed and rounded ends to the long and heavily beaded variety. Many of the latter showed four to five beads of chromatin (figure 7).

There was no question that multiplication had taken place, but what should especially be emphasized is that in this short sojourn in the body the morphology of the organism had so completely changed. The peritoneal cavity of the second snake contained no exudate; but films made from the omentum and gut showed large numbers of acid-fast bacilli. Very few short unbeaded bacilli were present; the greater number were long, thick, distinctly beaded, and pleomorphic. Many large globi and bacterial masses occurred. The most striking feature again was the morphological changes of the bacillus.

Experiment VII.—Two gold fish (*Carassius auratus*) received subcutaneous injections of the suspension of *B. lepræ*. The culture was one recently isolated from a case of human leprosy and still retained its long, slim, beaded appearance, and did not differ in morphology from the bacillus found in human leprous lesions. Its multiplication was very slow in the culture tube, in which it had been growing for two months when used in this experiment.

The first fish was examined at the end of twelve days, at which time it gave no evidence whatever of infection. Films from the point of inoculation, and at some distance within the muscle tissue, showed a few small masses of acid-fast granular detritus, and a few long and beaded bacilli similar to those injected, and other scattered ones in transition towards the small unbeaded and coccoid forms. The morphology, therefore, was changing from the type in human leprous lesions to the short unbeaded and coccoid forms of the leprosy bacillus grown *in vitro*. The second fish was killed thirty days after inoculation; it appeared normal. No lesions were found, but scattered and small clumps of bacilli were present. A few were contained inside of cells. Multiplication was evident, and the organisms were chiefly of the short, coccoid variety.

THE TRANSMISSION OF THE BACILLUS FROM FISH TO FISH.

Experiment VIII.—Three gold fish were inoculated below the skin of the back with 0.5 c.c. of an emulsion made from the tissues of a gold fish killed twenty-three days after primary inoculation. One died three days later, showing nothing of note.

One was killed thirty days after inoculation and several small opaque points, barely 0.5 mm. in diameter, appeared over the area covered by the injected fluid. The areas were superficial, and adhered to the skin. Films from the areas showed many cells resembling lepra cells containing organisms. The bacilli present were single and in small clumps of from six to ten. They all presented the characteristic short and unbeaded morphology.

The third fish was killed forty days after inoculation. External or internal evidence of infection was absent, the condition resembling the previous description.

THE TRANSMISSION OF BACILLUS LEPRÆ TO FISH BY WATER CONTAMINATED WITH THE ORGANISM.

Experiment IX.—Four day old slant cultures of the short unbeaded forms of *B. leprœ* were washed with sterile water and poured into an aquarium containing three gold fish, which were allowed to remain in the infected medium for seven days. They were then washed with several changes of water and transferred to a clean aquarium. The water was changed daily and the feces of the animals were examined every second or third day for acid-fast bacilli; thirteen days after the animals had been changed to uncontaminated water, the feces were found free from bacilli. Seventeen days after they had been removed from the source of infection, the first animal was killed. External evidences of infection were absent. The gills, as well as the skin and muscle tissue, were carefully examined for lesions or leprosy bacilli, but none were found. However, the omentum was covered with a gelatinous exudate, which contained large numbers of mononuclear cells and a few scattered acid-fast bacilli. The kidneys contained lepra bacilli, but none were found in the other organs. The organisms were short, unbeaded, and coccoid, and some showed deep staining bipolar granules, while a few were spindle-shaped.

The second fish was killed twenty-nine days, and the third fish thirty-eight days after inoculation. The appearances and the findings in these were similar to the previous one, except that there were probably a few more bacilli present in the latter.

THE TRANSMISSION OF BACILLUS LEPRÆ TO FISH BY FEEDING HUMAN LEPROSY NODULES AND THE FLESH OF INFECTED FISH.

Experiment X.—Two gold fish were fed teased nodules removed from a leper. No other food was allowed the animals until they had disposed of the leprosy tissue, after which they were cleansed and transferred to a clean aquarium.

The first was examined twenty-four days later; acid-fast organisms had not been found in the feces for three days before the animal was killed. No evidence of infection was apparent externally or internally. Films made from the omentum and other organs showed a few scattered acid-fast bacilli. The average number of bacilli to the slide was about four, and no change in morphology was seen. The second fish was examined after thirty-seven days. The findings were similar to those in the first one.

Experiment XI.—Bits of flesh from salt water fish infected with *B. leprœ* were fed to two gold fish. Three days after they had eaten the food, they were washed and transferred to a clean aquarium.

The first fish was killed twenty days later, and two days after the feces had been found free from acid-fast organisms. There was no microscopic evidence of lesions. A few scattered acid-fast bacilli were found in the omentum and other viscera. They were chiefly short and coccoid in form, although a few were slightly longer and showed distinct polar staining chromatin masses.

CONTROL EXPERIMENTS.

The possibility suggested itself that cold-blooded animals might harbor an acid-fast organism similar in appearance to the leprosy

bacillus. One or more specimens of each species was killed and examined in sections, and films were made from all the tissues, in order to find such an organism, if present. Moreover, the tap water and the food used for the fish were carefully controlled and often examined. At no time was it possible to demonstrate an organism which could have been confused with the leprosy bacillus used in this study.

SUMMARY AND DISCUSSION.

Before proceeding to a discussion of the experiments upon cold-blooded animals, it is necessary to review briefly some of the work recently done with the bacillus of leprosy. The appearance of the bacillus in man and its behavior under artificial cultivation, and in the tissues of lower animals, should be considered in order that comparisons may be drawn.

In their studies with the organism under cultivation, Duval and Gurd[2] pointed out that the long, slender, and beaded appearance of the leprosy bacillus described by Hansen,[3] in 1872, is lost when removed for several generations from the parent stem, and under artificial cultivation the organism becomes unbeaded, short, and coccoid. Duval also noted that these changes in morphology were always followed by rapid multiplication of the organism. Duval argues, *a priori,* that the bacillus is not in a favorable environment in the human tissues. If these deductions are correct, the morphology of the leprosy bacillus should vary according to the resistance offered by the tissues of different animals.

The resistance of the human host to the leprosy bacillus becomes more evident in the light of the clinical aspect of the disease. The long period of incubation, the duration of the disease, and the disappearance of the bacilli preceding the healing of the infected foci show that the resistance offered to the bacillus by the human tissues is not to be overestimated. This opinion is confirmed when the behavior of the leprosy bacillus under cultivation and in the tissues of various mammals is compared.

When cats, rabbits, bats, guinea pigs, and rats are inoculated either below the skin or into the peritoneal cavity with large quanti-

[2] *Arch. Int. Med.*, 1911, vii, 230.
[3] *Norsk. Mag. f. Laegevidensk.*, 1872, ii, 1.

ties of *Bacillus leprœ,* a slight local reaction follows within twenty-four to forty-eight hours, but no definite lesions are produced and the bacilli soon disappear. The resistance of some animals to *Bacillus leprœ* is well illustrated by two cats which were inoculated subcutaneously and intraperitoneally with a heavy suspension of *Bacillus leprœ.* These animals were killed and examined three days later, but the bacilli were not demonstrable from the regions about the sites of inoculation.

Pigeons are likewise refractory. It is impossible to cause a local reaction in these birds, and the injected bacilli disappear rapidly. Hence, probably no multiplication takes place in them.

Goats, young pigs, and white and dancing mice are in a degree susceptible to injections, and though undoubted lesions are produced, and multiplication of the bacilli occurs, the lesions and bacilli disappear after a limited time. Acid-fast bacilli which are recovered from the lesions are long, slim, and beaded, though the organisms used in the inoculations were short, unbeaded, and coccoid.

Monkeys inoculated with cultures of the short unbeaded forms react promptly. The lesions resulting, though confined in most instances to the site of inoculation, occasionally appear at distant points. The number of bacilli present in the nodules and their arrangement within typical lepra cells show that multiplication has taken place. The organism has, however, changed from the short coccoid form to the long, slender, beaded form. Though the lesions induced and the bacilli present are in every way similar to those found in man, their tendency to disappear gradually after a quiescent stage clearly denotes that the tissues of the monkey, although less refractory than the tissues of the animals previously mentioned, still offer resistance to invasion.

While mammals react but poorly to inoculations of the leprosy bacillus, this reaction manifests itself in various ways in different species. For example, while multiplication of the organism with the production of lesions occurs in some species, in others that are more refractory, the injected bacilli assume the involuted or beaded forms and do not multiply or produce lesions; in others, still more resistant to the action of the leprosy bacillus, the organisms quickly undergo granular metamorphosis and disappear. Furthermore, in

some species the lesions are, in most instances, limited to the site of inoculation, and though presenting all the characteristics of the lesion in man, the nodules and the bacilli disappear after a variable time. This behavior of the leprosy bacillus can be accounted for only by the degree of resistance offered by the tissues of the individual host.

Since the morphology of the organism invariably changes from the short coccoid to the large beaded form when placed in insusceptible animals, and conversely, from the long beaded forms to the short coccoid forms when placed in susceptible animals, the deduction can be drawn that the organism varies in morphology and rapidity of growth according to the susceptibility of the host.

Examples of similar behavior of *Bacillus leprœ* in the human subject are known to all investigators of leprosy. Ulcers and nodular areas often heal, and the bacilli disappear with little or no treatment. It is true that while older lesions are healing, new ones are constantly appearing, yet the duration of the disease and its undoubted tendency towards healing shows that conditions in the human subject are variable, and suggests that the organism has its natural habitat in some other host.

The experiments presented here serve to show that the bacillus of leprosy meets but little or no resistance in the tissues of cold-blooded animals, multiplies in their tissues, and may be harbored by them without apparent discomfort or external evidence of the disease.

That no appreciable resistance is offered to the multiplication of the leprosy bacillus by many species of cold-blooded animals is shown by the fact that aside from the trauma produced by the inoculation and the slight initial reaction of the tissues, the organism continues to grow profusely, and to invade the tissues without further reaction. Quite the opposite condition occurs in mammals: in some of these the leprosy bacillus degenerates into a granular mass shortly after inoculation; in others that are less refractory, typical lesions appear, but they seldom extend from the point of inoculation; and while the bacilli multiply slowly, they do not infiltrate the tissues, but disappear after a short time, the lesions healing.

That multiplication of *Bacillus leprœ* occurs in the tissues of cold-

blooded animals is shown by the fact that while animals examined a few days after inoculation show but a few scattered organisms, those killed at longer intervals show a proportional increase in the number of bacilli. Furthermore, the few bacilli found at the early period are extracellular and scattered, while after longer periods they tend to be massed and enclosed in large lepra cells.

The supposition that these lepra cells are phagocytes has naturally arisen. Duval[4] holds that they are not phagocytes in the true sense of the term, that the bacilli penetrate the cells rather than that the cells engulf them, after which, finding conditions for growth favorable, they multiply without causing serious injury to the cell. The size of the cell depends upon the size of the colony within. The experimental work bears out this view since the decrease in number of the organisms observed in animals killed shortly after inoculation depends not upon phagocytic action nor upon cells which appear later when active lesions are established. In early lesions, the lepra cells are smaller, barely measuring twenty to thirty microns in diameter, and contain but few bacilli; whereas in older ones, they attain a diameter of 100 microns or even more, and contain enormous numbers of bacilli. Were this increase in size due to phagocytic action, some cells would be found in which the limit of their capacity had been reached; and they would either contain a mass of dead and disintegrated bacteria or would themselves show evidence of disintegration. On the contrary, the bacilli, though they occupy most of the cell, show no signs of disintegration, and the nucleus and the cytoplasm of the cell retain normal staining properties. That the invasion and multiplication of the bacilli cause an irritation is evident by the amitotic divisions of the nucleus which occur in the larger cells.

The absence of external evidence of invasion by *Bacillus leprœ* in cold-blooded animals, and the apparent lack of discomfort caused by the presence of the organism within their tissues, are points which should be remembered in considering the sources from which leprosy may be transmitted. In not a single instance in the numerous experiments presented here would it have been possible, from

[4] *Jour. Exper. Med.*, 1910, xii, 649.

any external sign, to suspect that the animals were harboring multitudes of leprosy bacilli.

While the evidence in support of the opinion that leprosy may be transmitted from man to man appears sufficiently strong to warrant this belief, the number of cases in which infection can be actually traced to this source is small. Since leprosy is known to be prevalent where fish and sea-food are plentiful, and since the experiments here recorded prove that fish can be infected by being fed cultures of *Bacillus leprœ*, or nodules from human lepers, or bits of fish previously infected with the leprosy organism, account should be taken of the possibility that leprosy, in certain localities, may arise from this source of infection.

The question as to how and from what source leprosy bacilli enter the human body may be still regarded as an open one. Isolated examples of direct infection of healthy human beings from lepers have been reported by Arning and Nonne,[5] by Manson,[6] and others. The notion that the agency of infection is already infected human beings, that is lepers, is at the foundation of the modern practice of the isolation and segregation of lepers, which would seem to have brought about a definite decrease in the prevalence of the disease. It is an acknowledged fact, however, that the lepers confined in institutions practically never cause infection of nurses, etc. Some other factor than the human agency may therefore be considered as affecting this issue.

It is well known that Jonathan Hutchinson[7] has brought forward the idea that fish are the source of the infection, basing the view on the high prevalence of the disease along the coast countries of Norway and Sweden, and in the Pacific Islands, and in the countries bordering the Mediterranean and Black Seas, in all of which fish furnish the chief food material. No convincing proof was ever adduced in support of this contention. But now that it has been shown that the leprosy bacillus survives and multiplies in cold-blooded animals, at least at room temperature in a warm climate,

[5] *Arch. f. path. Anat.*, 1893, cxxxiv, 319.

[6] Tropical Diseases, 1900, 448.

[7] *Verhandl. d. X. internat. med. Congr.*, Berlin, 1890, iv, Abt. 13, p. 42.

and since methods have been devised for cultivating and identifying the leprosy bacillus, the question has been opened up to accurate investigation.

Duval has shown that the leprosy bacillus in cultures grows better at room temperature than at 37° C., so that growth in cold-blooded animals kept at room temperature is perhaps in some way connected with this phenomenon. What must now be ascertained, in order to test the Hutchinsonian theory more accurately is whether such growth takes place at a temperature corresponding with the average mean temperature of such a body of water as the North Sea and that of the fiords of Norway.[8] Since cold-blooded animals possess the same temperature as their surroundings, they would be suitable media for the cultivation of leprosy bacilli at those temperatures. For the waters of the Mediterranean Sea and the tropical Pacific Ocean, this consideration would count less. But the theory will stand or fall according as it can account for the whole, and not only for a part, of the phenomena to be explained.

As the length and shape of the bacilli and the number of chromatin masses are constant for a given species of cold- or warm-blooded animals, which features are governed by the resistance of the individual species, the following conclusions seem justified: that the morphology and rapid multiplication of the leprosy bacillus in cultures and in some species of cold-blooded animals indicate that *Bacillus lepræ* under natural conditions is short, coccoid, and unbeaded, and that the long, slender, beaded variety which occurs in the mammalian species is atypical and the product of an unfavorable environment.

In conclusion, I wish to thank Dr. C. W. Duval for valuable suggestions during the course of this work.

EXPLANATION OF PLATE LXXVIII.

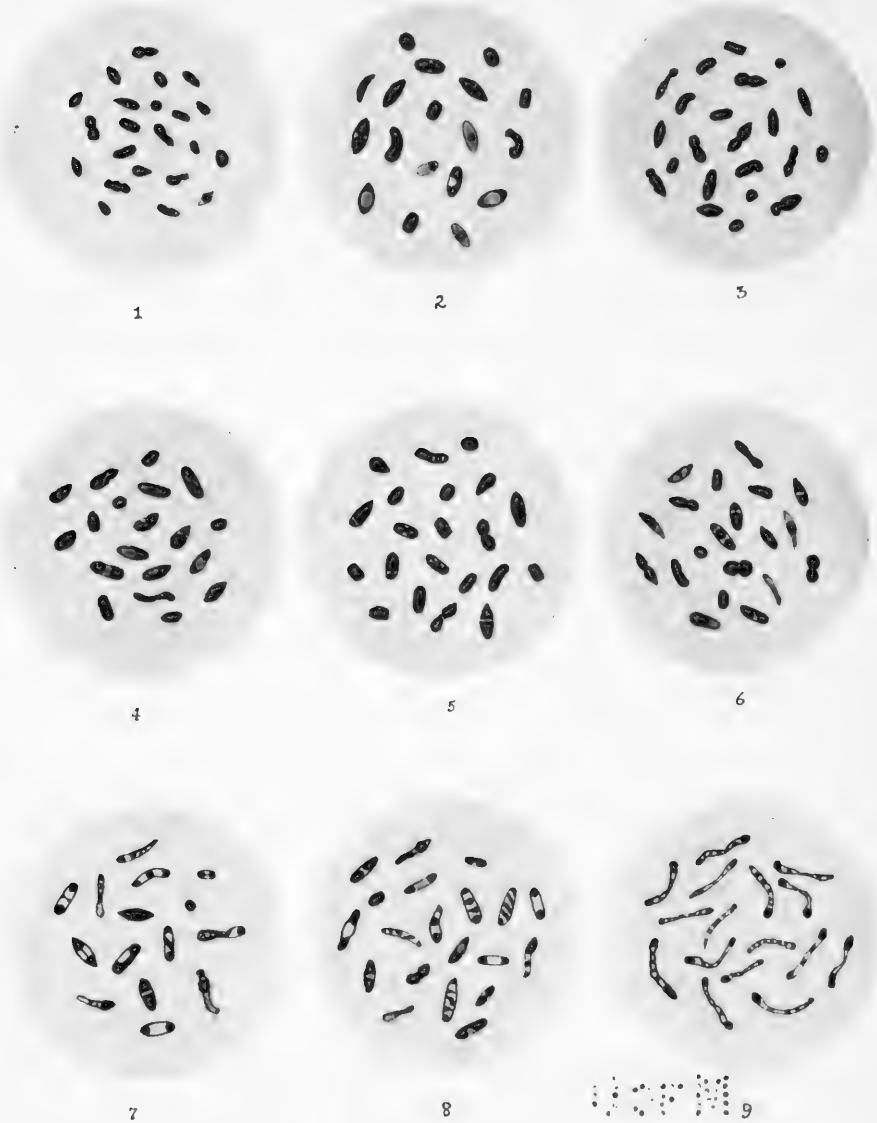
FIG. 1. Leprosy bacilli from the culture used in these experiments, growing upon fish agar. Note the short, unbeaded, coccoid forms.

FIG. 2. Bacilli from the same stem as figure 1, cultivated on alkaline glycerine agar.

FIG. 3. Leprosy bacilli from a gold fish killed thirty-nine days after inoculation.

FIG. 4. The bacillus in the frog thirty days after inoculation.

[8] Duval has recently found that *Bacillus lepræ* multiplies readily at 10° C.



1 2 3

4 5 6

7 8 9

FIG. 5. *Bacillus leprœ* in turtles thirty-five days after inoculation.

FIG. 6. Leprosy bacillus in tadpoles killed twenty-eight days after inoculation.

FIG. 7. The organisms in the king snake twelve days after inoculation.

FIG. 8. *Bacillus leprœ* in salt water fish, showing the changes in morphology fourteen days after inoculation. Note the pleomorphic appearance of the organisms.

FIG. 9. The appearance of the bacillus in human tissues and in cultures recently isolated from leprous lesions of man and other mammalian species.

COMPLEMENTOID AND THE RESISTANCE OF THE MID-PIECE OF COMPLEMENT.*

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With the discovery that the haptophore and toxophore groups of complement might be isolated, these two fractions have lately been subjected to direct analysis as to their thermic resistance, with the object of obtaining a clearer insight into complementoid action. Ferrata,[1] who made the first observation, claimed that the haptophore group (the so-called mid-piece) of complement, is thermostabile, resisting heating at 55° C. for one half hour, while the toxophore group (the so-called end-piece) is non-resistant. Hecker[2] and Tsurusaki[3] have been unable to verify Ferrata's results, reporting that both groups are equally thermolabile.

Since none of these experiments were conducted quantitatively, it seemed advisable to go over the ground again from this standpoint. But in spite of the most varied quantitative relationships, in no case were we able to demonstrate thermostability of the isolated haptophore group. Our results, therefore, confirm and amplify the finding of both Hecker and Tsurusaki.

Since the isolated haptophore group has been shown to undergo certain changes which do not occur when it is not separated from native serum, its behavior in the isolated condition may be regarded, in a certain sense, as abnormal. Our next point of attack was, therefore, to learn whether under more normal conditions its thermostability might not be demonstrated.

The method of investigation was simple. Guinea pig serum was inactivated by heating in the water bath at 55° C. for from twenty

* Received for publication, March 20, 1911.

[1] Ferrata, *Berl. klin. Wchnschr.*, 1907, xliv, 366.

[2] Hecker, *Arb. a. d. k. Inst. f. exper. Therapie zu Frankfurt a. M.*, 1907, No. 3, 39.

[3] Tsurusaki, *Biochem. Ztschr.*, 1908, x, 345.

minutes to one hour, and its inactivity demonstrated by directing it against sheep corpuscles laden with ten immune amboceptor units. To diminishing amounts of such inactive serum, a constant amount of guinea pig end-piece was now added, the inactivity of this complement fraction in itself having been previously determined and carefully controlled in the experiment proper. The results are of considerable interest since they demonstrate for the first time by direct experiment that the haptophore group of complement is thermostabile.

The sera examined may be divided into two groups according to their thermoresistance. One group was not completely inactivated after heating for twenty minutes, while the other group was inactivated. After heating for thirty minutes, however, both groups showed inactivity. These variations in thermic resistance are mentioned because of the difference in effect of more prolonged heating on haptophore activity. In the first instance, for example, prolonged heating was found to have considerably less destructive action than in the second, though in both instances the heated complement showed no activity after heating for thirty minutes. The following tables make this clear.

TABLE I.

Guinea Pig Serum Still Active after 20 Minutes Heating; Inactive after 30 Minutes Heating at 55° C.

In the following experiments 5 per cent. of sheep corpuscles and ten immune amboceptor units were used. I, II, III, and IV indicate hemolysis with guinea pig serum heated at 55° C. for 30, 40, 50, and 60 minutes respectively. Incubation time, one hour in water bath at 37° C. The maximum hemolytic activity was reached at the end of a half hour.

Guinea pig end-piece.	Heated guinea pig serum.	Hemolysis.			
		I	II	III	IV
—	.5 c.c.	0	0	0	0
—	.3 c.c.	0	0	0	0
—	.2 c.c.	0	0	0	0
.1 c.c.	—	0	0	0	0
.1 c.c.	.5 c.c.	Complete	Complete	Slight trace	0
.1 c.c.	.3 c.c.	"	"	Trace	0
.1 c.c.	.2 c.c.	"	"	Moderate	0
.1 c.c.	.1 c.c	"	"	"	Slight trace
.1 c.c.	.08 c.c.	"	"	Little	0
.1 c.c.	.06 c.c.	"	"	Very little	0
.1 c.c.	.04 c.c.	"	"	0	0
.1 c.c.	.02 c.c.	Almost complete	Little	0	0
.1 c.c.	.01 c.c.	Trace	Slight trace	0	0

TABLE II.

Guinea Pig Serum Inactive after 20 Minutes Heating at 55° C.

The same method was used as in Table I. I, II, III, and IV indicate hemolysis with serum heated for 20, 30, 40, and 60 minutes respectively.

Guinea pig end-piece.	Heated guinea pig serum.	Hemolysis.			
		I	II	III	IV
—	.5 c.c.	0	0	0	0
—	.3 c.c.	0	0	0	0
—	.2 c.c.	0	0	0	0
.1 c.c.	—	0	0	0	0
.1 c.c.	.5 c.c.	Complete	Complete	Little	0
.1 c.c.	.3 c.c.	"	"	Moderate	0
.1 c.c.	.2 c.c.	"	"	"	0
.1 c.c.	.1 c.c.	"	Almost complete	"	0
.1 c.c.	.08 c.c.	Almost complete	"	Little	0
.1 c.c.	.06 c.c.	Very strong	Strong	Trace	0
.1 c.c.	.04 c.c.	Strong	Little	Slight trace	0
.1 c.c.	.02 c.c.	Little	Very little	0	0
.1 c.c.	.01 c.c.	Trace	Slight trace	0	0

It is interesting to note that while 0.5 cubic centimeter of heated guinea pig serum gives no trace of hemolysis, as little as 0.04 cubic centimeter of this same serum on the addition of end-piece (toxophore group) inactive in itself, leads to complete hemolysis. It will be further seen (table I) that even after heating for forty minutes at 55° C. there may be no diminution in haptophore completing action. In most cases, however, the decrease after fifty minutes was marked, while after sixty minutes the completing action was practically nil.

We have also examined one guinea pig complement inactive after standing for seventeen days at room temperature. This serum, though devoid of hemolytic activity even in the presence of ten amboceptor units, likewise showed a strong completing action on the addition of guinea pig end-piece, the addition of 0.03 cubic centimeter of inactive serum still sufficing to produce complete hemolysis.

The results given above may be criticised on the ground that although hemolysis occurs in the combination inactivated complement + end-piece, it has not been shown that this completing action is due to a thermoresistant mid-piece. To settle this point, therefore, the globulin fraction, which, as is known, contains the mid-piece, was precipitated from guinea pig serum inactivated by heat,

and against this the end-piece was directed. The globulin precipitates were obtained by the hydrochloric acid and carbon dioxid methods and were carefully washed with distilled water to free them as far as possible from all traces of serum. To diminishing amounts of such mid-piece, a constant amount of end-piece was now added, ten amboceptor units and 5 per cent. sheep corpuscles being used as above.

The results (table III) show that such a mid-piece isolated from inactivated serum may exert a completing action as great or even greater than the completing action of whole inactivated serum. A closer study of the relative completing strength of whole inactive serum and the globulin fractions of unheated and heated serum is now being made.

TABLE III.

Guinea Pig Serum Heated for 40 Minutes at 55° C. Globulin Fraction Obtained by N/250 Hydrochloric Acid. Incubation Time in Water Bath One Hour.

Guinea pig end-piece.	Heated guinea pig serum.	Hemolysis.	Hemolysis.	Mid-piece from heated guinea pig serum.	Guinea pig end piece.
.1 c.c.	—	0	0	—	.1 c.c.
—	1.0 c.c.	0	0	1.0 c.c.	—
.1 c.c.	1.0 c.c.	Complete	Complete	1.0 c.c.	.1 c.c.
.1 c.c.	.5 c.c.	"	"	.5 c.c.	.1 c.c.
.1 c.c.	.25 c.c.	"	"	.25 c.c.	.1 c.c.
.1 c.c.	.1 c.c.	"	"	.1 c.c.	.1 c.c.
.1 c.c.	.08 c.c.	Almost complete	Almost complete	.08 c.c.	.1 c.c.
.1 c.c.	.06 c.c.	Very strong	" "	.06 c.c.	.1 c.c.
.1 c.c.	.04 c.c.	Strong	Strong	.04 c.c.	.1 c.c.
.1 c.c.	.02 c.c.	Little	Little	.02 c.c.	.1 c.c.
.1 c.c.	.01 c.c.	Trace	Trace	.01 c.c.	.1 c.c.

A discussion of the mechanism of these phenomena must be left for a future communication. The activation of complementoid by a toxophore group is hard to understand on the Ehrlich hypothesis, since it is assumed that in complementoid the haptophore group is still possessed by an inert toxophore group. To explain this matter it would have to be assumed that the inert toxophore group is set free and that the fresh end-piece, on account of its greater avidity, then seizes upon the haptophore group. Whether the two portions of the complement exist combined or dissociated in native serum, must still be left open to investigation, since we have been able to

show in connection with the above work, that in the system, sheep corpuscles + sheep-rabbit amboceptor + active sheep serum + guinea pig end-piece, hemolysis may occur. Whether this signifies that the complement of the sheep serum becomes dissociated in the presence of sensitized corpuscles or whether its haptophore and toxophore groups exist side by side, can not be stated at present.